

**REMARKS**

***Regarding the amendments***

The specification has been amended at page 4 to replace the first full paragraph with a paragraph which is identical, except for replacing the abbreviation "SNAP-25" at line 12, with the corresponding full name "synaptosome-associated protein of molecular weight 25 kilodaltons." The amendment to page 4 of the specification is supported, for example, by Vaidyanathan et al., J. Neurochem. 72: 327-337 (1999), at page 327, abstract, which indicates that the term "SNAP-25" is an abbreviation for "synaptosome-associated protein of 25 kDa." Vaidyanathan et al., which is cited in the specification at page 30, lines 26-27, and incorporated by reference into the specification at page 116, lines 21-24, is attached hereto as Exhibit A.

The specification has similarly been amended at page 5 to replace the first incomplete paragraph with a paragraph which is identical, except for replacing the term "VAMP" at line 8 with the corresponding full name "vesicle-associated membrane protein." The amendment to page 5 of the specification also is supported, for example, by Vaidyanathan et al., Exhibit A, page 327, abstract, which indicates that the term "VAMP" is an abbreviation for "vesicle-associated membrane protein."

Claim 42 has been amended to indicate that the recited sample is formulated BoNT/A product containing human serum albumin. The amendment to claim 42 is supported throughout the specification, for example, at page 104, lines 20-28, which

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discloses formulated clostridial toxin products containing human serum albumin, and in Example I (especially page 115, lines 29-31), which indicates that the formulated BOTOX® product is a BoNT/A.

Claims 49 to 52 have been amended herein to recite a "BoNT/A or BoNT/E substrate" in order to agree with the antecedent basis provided in base claim 35 (step a). The amendment to these claims is supported by original claim 35 and does not add new matter.

As set forth above, each of the amendments to the specification and claims is supported by the specification or claims as originally filed and does not add new matter. Accordingly, Applicants respectfully request that the Examiner enter the amendments.

***Regarding Objections to the Specification***

**Regarding "SNAP" and "VAMP"**

The Examiner objects to the specification for using the abbreviations SNAP, VAMP and FLAG. As discussed above, the specification has been amended herein at pages 4 and 5 to give the full name for the abbreviations "SNAP" and "VAMP" where they appear for the first time in the specification.

**Regarding "FLAG"**

To Applicants' knowledge, "FLAG" is not an abbreviation but is, instead, a nonproprietary designation for a

particular, well-known 8-residue epitope. Applicants respectfully point out that such names used in trade are permissible in patent applications if, in this country, their meanings are well known and satisfactorily defined in the literature as set forth in MPEP 608.01(v). As evidence that the term "FLAG" was well known and defined at the time the subject application was filed, Applicants submit herewith Exhibits B and C. As indicated in Exhibit B (Brizzard et al., "Immunoaffinity Purification of FLAG epitope-tagged bacterial alkaline phosphatases using a novel monoclonal antibody and peptide elution," Biotechniques 16:730-735 (1994)), the FLAG epitope is an eight amino acid peptide which has the sequence Asp Tyr Lys Asp Asp Asp Asp Lys and is useful for immunoaffinity purification (see abstract). Similarly, Exhibit C (Fulton et al., "Functional analysis of avian class I (BPIV) glycoproteins by epitope tagging and mutagenesis in vitro," Eur. J. Immunol. 25: 2069-2076 (1995)), corroborates that "FLAG" was a well-known epitope tag (page 2069, abstract). In light of these 1994 and 1995 publications, Applicants submit that "FLAG" was a well-defined term in the art of molecular biology at the time the subject application was filed. Accordingly, Applicants submit that the use of the term "FLAG" is appropriate in the subject application.

In view of the above, Applicants respectfully request that the Examiner remove the objection to the specification based on the use of the terms "SNAP," "VAMP," and "FLAG."

Regarding several trademarks

The use of several trademarks has been noted by the Examiner, for example, at pages 4 to 11, 14 and 20 of the specification. The Office Action indicates that, while the use of trademarks is permissible in patent applications, the proprietary nature of the marks should be respected and every effort made to prevent their use in any manner that might adversely affect their validity as trademarks. The Office Action further indicates that trademarks should be capitalized and accompanied by generic terminology.

Applicants respectfully submit that trademarks have not been used in the specification in a manner which would adversely affect their validity. Rather, the proprietary nature of the marks have been respected by the use of the proper trademark symbol “®” following the name of the trademark. See MPEP 608.01(v). Thus, Applicants have respected the proprietary nature of trademarks such as Alexa Fluor® 488, QSY 7® and BOTOX®. Applicants further submit that no accompanying generic terminology is necessary where the trademark has a fixed and definite meaning in the art, unless some characteristic of the trademarked article is involved in the invention (MPEP 608.01(v)). In view of the above remarks, Applicants submit that trademarks have been properly used in the specification, and respectfully request that this ground for objecting to the specification be removed.

***Regarding the Objections to the Claims***

The objection to claims 39 and 40 under 37 C.F.R. 1.75(c) as allegedly in improper multiple dependent claim form is respectfully traversed.

Applicants respectfully disagree that the wording of claims 39 and 40 is improper and assume, absent any indication to the contrary, that the objection is based on the withdrawal of claim 37. Applicants respectfully request that the Examiner withdraw the embodiment of each of claims 39 and 40 depending from claim 37, and remove the objection as it pertains to the remaining embodiments of claims 39 and 40. Should this objection be maintained, further clarification by the Examiner is respectfully requested.

***Regarding the double patenting rejections***

The provisional rejection of claims 35, 36, 38, 39, 41, 42, 44, 45, 47, 48 and 53 under the judicially created doctrine of obviousness-type double patenting as allegedly unpatentable over claims 61 to 63, 67, 71 to 74, and 76 to 82 of U.S. application Serial No. 10/261,161 is respectfully traversed. Applicants respectfully defer responding to this provisional rejection until allowable subject matter is indicated.

***Regarding the rejection under 35 U.S.C. § 112, first paragraph***

The rejection of claims 49 to 51, and corresponding objection to the specification under 35 U.S.C. § 112, first paragraph, as allegedly failing to provide an enabling disclosure, respectfully are traversed. In this regard, while the Office Action acknowledges that "at most 5% cleavage" is enabled, it is alleged that the specification fails to enable "at most 15% cleavage," "at most 25% cleavage" and "at least 90% cleavage" of the recited substrate. The Office Action indicates that the rejection is based, in part, on the lack of working examples in the specification.

Applicants wish to clarify for the record that the recited "at most 5% cleavage," "at most 15% cleavage," "at most 25% cleavage" and "at least 90% cleavage" refer to the total percentage of the original amount of input substrate which is cleaved at the time resonance energy transfer is determined. In particular, claims 49 to 52 do not relate to hydrolysis rates, as asserted in the Office Action. Furthermore, Example I does not disclose a percentage of substrate cleaved but, rather, indicates that there is a reduction of at least about 5% in the emission wavelength at 585 nm, or an increase of about 5% in the emission wavelength at 520 nM, which is indicative of protease activity.

Applicants submit that, in view of the guidance provided in the specification, one skilled in the art would have been able to choose suitable conditions such that the desired extent of cleavage would be achieved in the protease assays of

the invention. In this regard, the specification teaches that assay times can be varied to achieve different extents of cleavage in a protease assay, for example, depending on the concentration, purity and activity of clostridial toxin in the sample (page 105, lines 14-22). Furthermore, as taught in the specification, assay times can be varied by convenient termination of the protease reaction by any of a variety of means such as addition of  $H_2SO_4$ , sodium borate, high pH or zinc chelators (specification at page 105, lines 22-28), or by varying the time at which resonance energy transfer is determined. Thus, the extent of cleavage in a protease assay of the invention can be routinely adjusted by varying assay times, for example, using well-established termination conditions

In view of the above, only routine techniques, and not undue experimentation, would have been required for the skilled person to adjust the extent of substrate cleavage depending, for example, on the purity and concentration of toxin in the sample and the reaction conditions selected. Guidance regarding specific exemplary conditions suitable for BoNT/A protease activity is provided throughout the specification, for example, at page 105, line 29, to page 106, line 14. Further, laboratory workers standardly vary assay parameters such as reaction length, and such standard variation of one or more assay parameters does not require undue experimentation.

In sum, in view of the guidance in the specification and what was well known in the art at the time the invention was made, varying assay times to adjust the extent of cleavage would have been well within the level of one skilled in the art and

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would not constitute undue experimentation. Accordingly, Applicants respectfully request that the Examiner remove the rejection of claims 49 to 51, and the corresponding objection to the specification under the first paragraph of 35 U.S.C. § 112.

***Regarding the rejection under 35 U.S.C. § 112, second paragraph***

The rejection of claims 35 to 53 under 35 U.S.C. §112, second paragraph, as allegedly lacking clarity respectfully is traversed.

**Regarding the phrase "under conditions suitable for clostridial toxin protease activity"**

Claim 35 recites "treating a sample, under conditions suitable for clostridial toxin protease activity, with a BoNT/A or BoNT/E substrate." This claim stands rejected under the second paragraph of 35 U.S.C. § 112 on the ground that the metes and bounds of such "suitable conditions" are unclear.

Applicants submit that the phrase "conditions suitable for clostridial toxin protease activity" is clear to the skilled person in view of the specification and what was well known in the art. In particular, the skilled person understands that proteases and other enzymes have activity under suitable conditions such as within a range of salt concentrations, range of pH, range of temperatures and in the presence of any necessary co-factors. Where a sample containing BoNT/A or BoNT/E toxin is treated with a BoNT/A or BoNT/E substrate "under

conditions suitable for clostridial toxin protease activity," the BoNT/A or BoNT/E substrate will be cleaved. In contrast, one skilled in the art understands that, even where a sample containing BoNT/A or BoNT/E toxin is treated with a substrate, there will be no cleavage under conditions which are not suitable for clostridial toxin protease activity.

Furthermore, the phrase "conditions suitable for clostridial toxin protease activity" is clear to the skilled person in view of the specification. Exemplary conditions suitable for clostridial toxin protease activity are well known in the art as set forth in the specification (page 102, lines 16-22). In this regard, the specification teaches that "conditions suitable for clostridial toxin protease activity generally include an appropriate concentration of a buffer such as HEPES, Tris or sodium phosphate;  $\beta$ -mercaptoethanol, dithiothreitol (DTT) or another reducing agent where the sample contains dichain toxin; and a source of zinc; and, further, generally exclude zinc chelators such as EDTA (page 102, line 22, to page 103, line 15). The specification further teaches that exemplary conditions "suitable for BoNT/A protease activity" can be incubation at 37°C in a buffer such as 30 mM HEPES (pH 7.3) containing a reducing agent such as 5 mM dithiothreitol; a source of zinc such as 25  $\mu$ M zinc chloride and 1  $\mu$ g/ml toxin, with bovine serum albumin (BSA) in the range of 0.1 mg/ml to 10 mg/ml (page 105, line 29, to page 106, line 6). As exemplified in Example I and taught in the specification, "conditions suitable for BoNT/A activity" also can be incubation at 37°C for 30 minutes in a buffer containing 50 mM HEPES (pH 7.4), 1% fetal bovine serum, 10  $\mu$ M zinc chloride and 10 mM DTT

with 10  $\mu$ M substrate (page 106, lines 9-14). In view of the above and what was well known in the art at the time the application was filed, one skilled in the art understands that "conditions suitable for clostridial toxin protease activity" are any of the above or similar conditions under which a clostridial toxin substrate is specifically cleaved. In sum, Applicants submit that the phrase "conditions suitable for clostridial toxin protease activity" is clear and definite, and, accordingly, respectfully request that the Examiner remove this ground for rejection.

Regarding the phrase "under the appropriate conditions"

The concluding phrase of claim 35, step (a), recites that "under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor." The Office Action alleges that the metes and bounds of such "appropriate conditions" are not clear. Applicants respectfully traverse this rejection of claim 35.

Applicants submit that, in view of the specification, one skilled in the art understands that resonance energy transfer can be exhibited between a particular donor fluorophore/acceptor pair. As is well known in the art and set forth in the specification, fluorescence resonance energy transfer is a physical process by which energy is transferred non-radiatively from an excited donor fluorophore to an acceptor through intramolecular long-range dipole-dipole coupling (specification at page 68, lines 21-26). As further taught in

the specification, actual resonance energy transfer is exhibited when the donor fluorophore is excited under appropriate conditions which include an appropriate separation distance and orientation of the donor fluorophore and acceptor as described by the Forster equation, and further depends in part on the fluorescent quantum yield of the donor fluorophore and its energetic overlap with the acceptor (page 68, lines 21-33; page 69, line 25, to page 70, line 71, line 12). Appropriate conditions also include substantial spectral overlap between the emission spectrum of the donor fluorophore and the excitation spectrum of the acceptor (page 71, lines 15-19).

In view of what was known about the phenomenon of resonance energy transfer and the teachings of the specification, it is clear to the skilled person that "appropriate conditions" for resonance energy transfer include excitation of the donor fluorophore; selection of a donor fluorophore/acceptor pair such that there is substantial spectral overlap between the emission spectrum of the donor fluorophore and the excitation spectrum of the acceptor; and an appropriate separation distance and orientation of the donor fluorophore and acceptor in the clostridial toxin substrate. In sum, Applicants submit that the clause "under the appropriate conditions, resonance energy transfer is exhibited between said donor fluorophore and said acceptor" is clear to the skilled person in view of the specification. Applicants therefore respectfully request that the Examiner remove this ground for rejecting claim 35 as allegedly indefinite.

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Regarding claims 39 and 40

Claims 39 and 40 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly unclear for reciting "wherein said sample is isolated clostridial toxin." Applicants submit that claims 39 and 40 are clear as written. Nevertheless, in order to further clarify this phrase, claims 39 and 40 have been amended to recite "wherein said sample is an isolated clostridial toxin." Introduction of the article "an" is made in order to further prosecution of the subject application and does not change the scope of the claims. Accordingly, Applicants respectfully request that the Examiner remove this ground for rejecting claims 39 and 40 under 35 U.S.C. § 112, second paragraph.

Regarding claim 42

Claim 42 stands rejected as allegedly unclear due to the use of the trademark BOTOX®. In making the rejection, the Office Action states that a trademark or trade name identifies a source of goods rather than the goods themselves and further indicates that a trademark or trade name cannot be used to identify a particular material or product in a claim.

Applicants submit that claim 42 is clear as written. Nevertheless, in order to further prosecution, claim 42 has been amended to delete the term "BOTOX®" and to specify that the sample is a formulated BoNT/A product containing human serum albumin. The amendment to claim 42 does not narrow the scope of

the claim. In view of the above remarks and amendments, Applicants submit that the scope of claim 42 is clear and definite and respectfully request that the Examiner remove this ground for rejection.

Regarding claim 53

In regard to claim 53, the Office Action asserts that the phrase "wherein the conditions suitable for clostridial toxin protease activity are selected such that the assay is linear" is not clear. The Office Action queries how one skilled in the art can select such conditions.

A "linear" assay is a term of art well-known to the skilled person to mean an assay in which there is a direct, or linear, relationship between two variables. In the present case, the methods of the invention are directed to determining protease activity of BoNT/A or BoNT/E by determining resonance energy transfer of a treated BoNT/A or BoNT/E substrate. Thus, it is clear to the skilled person that, where conditions are selected such that an assay of the invention is linear, there will be a direct relationship between the protease activity in the sample and the observed resonance energy transfer. Applicants therefore submit that the phrase "wherein the conditions suitable for clostridial toxin protease activity are selected such that the assay is linear" is clear and definite to the skilled person.

As further clarification regarding conditions suitable for "linear assays," the specification teaches that linearity is lost in assays run using high concentrations of substrate or toxin due to the "inner filter effect" involving intermolecular energy transfer. The specification further teaches that the concentration of clostridial toxin substrate or the amount of sample can be limited to produce a linear assay. As non-limiting examples, suitable conditions for linear assays include limiting clostridial toxin substrate concentrations to less than 100  $\mu$ M, 50  $\mu$ M or 25  $\mu$ M (page 104, lines 1-12). As further guidance to the skilled person regarding conditions suitable for linear assays, the specification teaches that a linear assay can be performed by diluting clostridial toxin substrate with corresponding unlabeled substrate lacking donor fluorophore and acceptor (page 104, lines 12-19). Similarly, purified or partially purified or crude toxin samples can be diluted to within a convenient range such that the assay for toxin protease activity is linear (page 104, line 30, to page 105, line 2). Thus, in view of the well known meaning of the term "linear assay" and further in view of the guidance in the specification, Applicants submit that the phrase "wherein the conditions suitable for clostridial toxin protease activity are selected such that the assay is linear" is clear and definite to the skilled person. Accordingly, Applicants respectfully request that the Examiner remove the rejection of claim 53 under 35 U.S.C. §112, second paragraph.

Regarding the terms "at most" and "at least"

It is alleged that the terms "at most" and "at least" in claims 49 to 52 are relative terms that render the claims indefinite.

Applicants submit that claims 49 to 52 are clear and definite as written. Claim 49 recites wherein at least 90% of said BoNT/A or BoNT/E substrate is cleaved; and claims 50 to 52 recite wherein at most 25%, 15% or 5%, respectively, of said BoNT/A or BoNT/E substrate is cleaved. From the plain meaning of "at least 90%" in claim 49, one skilled in the art understands that cleavage of 90%, 91%, 92%, 95% or more of the BoNT/A or BoNT/E substrate falls within the claims. In short, one skilled in the art understands that, in the method of claim 49, 90% or more of the BoNT/A or BoNT/E substrate is cleaved. It is further understood that the recited "90%" is relative to the original amount of uncleaved BoNT/A or BoNT/E substrate with which the sample was treated. Similarly, from the plain meaning of "at most 25%" in claim 50, one skilled in the art understands that cleavage of, for example, 1%, 5%, 10%, 15%, 20% or 25% of the BoNT/A or BoNT/E substrate is encompassed by claim 50. Clearly, in the method of claim 50, there is cleavage of 25% or less of the original amount of uncleaved substrate with which the sample was treated. Analogously, there is cleavage of 15% or less, or cleavage of 5% or less, respectively, of the original amount of uncleaved substrate in the method of claim 51 or 52. In sum, Applicants assert that one skilled in the art is unambiguously apprised of the scope of claims 49 to 52. The Examiner is therefore respectfully requested to remove the

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rejection of claims 49 to 52 under 35 U.S.C. §112, second paragraph.

Regarding the phrase "at one or more later time intervals"

The Examiner alleges that claim 48, which depends on base claim 35, is indefinite for reciting "repeating step (c) at one or more later time intervals."

Applicants submit that claim 48 is clear and definite in view of the specification. As is clear from reviewing the language of step (c) of base claim 35, the step which is to be repeated at one or more later time intervals is "determining resonance energy transfer of said treated substrate relative to a control substrate." From this language, one skilled in the art clearly understands that step (c) is performed two or more times. One skilled in the art further clearly understands that the one or more repetitions of step (c) are not performed simultaneously but, rather, are performed sequentially.

Claim 48 further is clear and definite in view of the teachings of the specification. The specification teaches, for example, that the methods of the invention for determining clostridial toxin protease activity involve determining resonance energy transfer of a clostridial toxin substrate treated with a sample relative to a control substrate and can be practiced as fixed-time or continuous-time assays. In a "fixed-time" assay, resonance energy transfer is determined at a single point in time, while in continuous-time assays, fluorescence

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resonance energy transfer is determined at two or more, five or more, ten or more, or twenty or more different intervals (page 112, line 26, to page 113, line 5). In sum, one skilled in the art understands that the method of claim 48 is practiced by determining resonance energy transfer at least twice, with the determinations occurring sequentially rather than simultaneously. Accordingly, claim 48 is clear and definite as written, and Applicants respectfully request that the rejection of claim 48 be removed.

***Regarding the rejection under 35 U.S.C. §103***

The rejection of claims 35 to 53 under 35 U.S.C. §103(a) as allegedly obvious over Schmidt and Bostian (U.S. Patent No. 5,965,699) in view of Clegg, Curr. Opin. in Biotech. 6:103-110 (1995), respectfully is traversed.

The Office Action indicates that the cited Schmidt and Bostian patent describes a label-based assay for quantitation of BoNT/A. In particular, Schmidt and Bostian appear to describe a 17-mer BoNT/A peptide substrate and a fluorescamine label for detection of free amino groups following proteolysis of peptide substrate by the toxin. The Office Action acknowledges that, while Schmidt and Bostian appear to describe the use of fluorescamine as a fluorophore, the cited patent does not teach or suggest assaying BoNT/A activity using fluorescence resonance energy transfer as claimed. However, it is alleged that Clegg generally reports the utility of fluorescence resonance energy transfer in biological assays and for the study of enzymes. The

Office Action concludes that it would have been obvious to combine the method of Schmidt and Bostian with the well known FRET technique reviewed in Clegg.

Applicants submit that the methods of the invention are unobvious over the cited references, either alone or in combination. Specifically, neither Schmidt and Bostian nor the cited Clegg publication teaches or suggests resonance energy transfer assays for determining protease activity of BoNT/A or BoNT/E and, further, does not teach or suggest a BoNT/A or BoNT/E substrate containing a donor fluorophore, an acceptor and a BoNT/A or BoNT/E recognition sequence including a cleavage site. Furthermore, as discussed below, there is no motivation to modify the assay of Schmidt and Bostian by incorporating fluorescence resonance energy transfer.

Specifically, the claimed resonance energy transfer-based methods are valuable, in part, because they are simple homogeneous screening assays that do not require separation of cleaved product from uncleaved substrate (specification at page 18, lines 20-25). However, this advantage is shared by the Schmidt and Bostian assay in which fluorescamine is used to label free amino groups in BoNT/A cleavage products. In this regard, Schmidt and Bostian report that:

We have developed a set of peptides containing modified sequences which do not react with fluorescamine but are good substrates for the proteolytic activity of type A botulinum toxin. Thus, the requirement for expensive and time-consuming product separation has been completely

eliminated (U.S. Patent No. 5,965,699;  
column 4, lines 21-25; emphasis added).

Schmidt and Bostian further report that outdated assays for BoNT/A activity relied on HPLC separation and quantitation of substrate hydrolysis products and further emphasized that their fluorescamine-based assay can be performed in an hour or less "since no separation of hydrolysis products is needed" (column 2, lines 20-26). Similarly, Schmidt and Bostian indicate that their fluorescamine-based assays are sensitive, accurate and "do not require prior separation of products" (column 2, lines 39-45). In view of these reported advantages, Applicants submit that one skilled in art would not have been motivated to modify the fluorescamine-based assay of Schmidt and Bostian, which was already sensitive and accurate and avoided product separation.

Furthermore, Schmidt and Bostian describe labeling a BoNT/A cleavage product with a fluorophore but do not teach or suggest a BoNT/A or other botulinum toxin substrate that contains a donor fluorophore or other fluorescent label. In particular, Schmidt and Bostian report labeling a hydrolyzed BoNT/A peptide product with the fluorophore fluorescamine. See, for example, Schmidt and Bostian at column 2, lines 37-41, which indicates that results were quantitated by addition of a label after toxin-catalyzed hydrolysis of peptide substrates, followed by measuring the amount of label; and column 4, lines 12-17, which indicates that the non-fluorescent detection reagent fluorescamine reacts with amino groups to give intensely fluorescent compounds. Thus, in the methods of Schmidt and Bostian, fluorescamine is used to label cleaved peptide product.

Given that cleaved peptide product, and not substrate, is labeled, the assay of Schmidt and Bostian is free from the possible interference with proteolysis that introducing a donor fluorophore into a substrate may produce. In contrast, one skilled in the art would not have known whether labeling a BoNT/A substrate with a bulky donor fluorophore would interfere with BoNT/A protease activity, and, therefore, would not have been motivated to modify the proven Schmidt and Bostian fluorescamine assay by incorporating a substrate that contains a donor fluorophore.

In sum, neither of the cited references, alone or in combination, teach or suggest a BoNT/A or BoNT/E substrate containing a donor fluorophore, an acceptor and a BoNT/A or BoNT/E recognition sequence which includes a cleavage site. Furthermore, there is no motivation to combine the teachings of Schmidt and Bostian with those of Clegg in order to modify Schmidt and Bostian's fluorescamine-based assay. Absent such motivation, the claimed methods are unobvious over the cited references.

In view of the above, Applicants respectfully request that the Examiner reconsider and remove the rejection of claims 35 to 53 under 35 U.S.C. § 103 over Schmidt and Bostian in view of Clegg.

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**CONCLUSION**

Applicants respectfully request that the Examiner consider the amendments and remarks herein above. Should the Examiner have any questions, he is invited to call the undersigned agent or Cathryn Campbell.

Respectfully submitted,

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Andrea L. Gashler

Andrea L. Gashler  
Registration No. 41,029  
Telephone: (858) 535-9001  
Facsimile: (858) 535-8949

McDERMOTT, WILL & EMERY  
4370 La Jolla Village Drive  
Suite 700  
San Diego, California 92122

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# Proteolysis of SNAP-25 Isoforms by Botulinum Neurotoxin Types A, C, and E: Domains and Amino Acid Residues Controlling the Formation of Enzyme-Substrate Complexes and Cleavage

Vadakkanchery V. Vaidyanathan, Ken-ichi Yoshino, Michael Jahnz, Christos Dörries, Steffen Bade, Sonja Nauenburg, Heiner Niemann, and Thomas Binz

Department of Biochemistry, Medizinische Hochschule Hannover, Hannover, Germany

**Abstract:** Tetanus toxin and the seven serologically distinct botulinum neurotoxins (BoNT/A to BoNT/G) abrogate synaptic transmission at nerve endings through the action of their light chains (L chains), which proteolytically cleave VAMP (vesicle-associated membrane protein)/synaptobrevin, SNAP-25 (synaptosome-associated protein of 25 kDa), or syntaxin. BoNT/C was reported to proteolyze both syntaxin and SNAP-25. Here, we demonstrate that cleavage of SNAP-25 occurs between Arg<sup>198</sup> and Ala<sup>199</sup>, depends on the presence of regions Asn<sup>93</sup> to Glu<sup>145</sup> and Ile<sup>156</sup> to Met<sup>202</sup>, and requires about 1,000-fold higher L chain concentrations in comparison with BoNT/A and BoNT/E. Analyses of the BoNT/A and BoNT/E cleavage sites revealed that changes in the carboxyl-terminal residues, in contrast with changes in the amino-terminal residues, drastically impair proteolysis. A proteolytically inactive BoNT/A L chain mutant failed to bind to VAMP/synaptobrevin and syntaxin, but formed a stable complex ( $K_D = 1.9 \times 10^{-7} M$ ) with SNAP-25. The minimal essential domain of SNAP-25 required for cleavage by BoNT/A involves the segment Met<sup>146</sup>–Gln<sup>197</sup>, and binding was optimal only with full-length SNAP-25. Proteolysis by BoNT/E required the presence of the domain Ile<sup>156</sup>–Asp<sup>186</sup>. Murine SNAP-23 was cleaved by BoNT/E and, to a reduced extent, by BoNT/A, whereas human SNAP-23 was resistant to all clostridial L chains. Lys<sup>185</sup>Asp or Pro<sup>182</sup>Arg mutations of human SNAP-23 induced susceptibility toward BoNT/E or toward both BoNT/A and BoNT/E, respectively. **Key Words:** Clostridial neurotoxins—SNAP-25—SNAP-23—Proteolysis—Protease binding assay.

*J. Neurochem.* 72, 327–337 (1999).

*Clostridia* produce several powerful neurotoxins; tetanus toxin and botulinum neurotoxins BoNT/A to BoNT/G cause the clinical manifestations of tetanus and botulism in a large variety of animal species and humans. The toxins are synthesized as single-chain polypeptides with molar masses of ~150 kDa. On lysis of the bacteria and activation by proteolytic cleavage, the light chains (L

chains; 50 kDa) remain disulfide bound to the heavy chains (H chains; 100 kDa). The extreme neurotoxicity is largely ascribed to the H chains, which bind to neuronal receptors that internalize the holotoxins, and translocate the L chains into the cytosol. Here, the L chains block fusion of synaptic vesicles with the presynaptic membrane (Simpson, 1989).

The genes of the eight known clostridial neurotoxins have been cloned and characterized (for review, see Niemann et al., 1994). The L chains contain a Zn<sup>2+</sup>-binding motif, His–Glu–X–X–His, also found in an increasing number of zinc-dependent metalloproteases (Jongeneel et al., 1989). Soon after demonstration of cleavage of VAMP (vesicle-associated membrane protein)/synaptobrevin (Trimble et al., 1988) by tetanus toxin and BoNT/B at the same peptide bond (Link et al., 1992; Schiavo et al., 1992), substrates and scissile bonds were identified for all other botulinum serotypes. These studies revealed that BoNT/D, BoNT/F, and BoNT/G also hydrolyze synaptobrevin, although each at a different peptide bond. BoNT/A and BoNT/E cleave SNAP-25 (synaptosome-associated protein of 25 kDa), again at distinct sites close to the C-terminus (for review, see

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Address correspondence and reprint requests to Dr. T. Binz at Department of Biochemistry, Medizinische Hochschule Hannover, Carl-Neuberg-Straße 1, D-30623, Hannover, Germany.

The present address of Dr. V. V. Vaidyanathan is Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

**Abbreviations used:** BoNT, botulinum neurotoxin; GST, glutathione S-transferase; GT, glutathione; L chain, light chain; NSF, N-ethylmaleimide sensitive fusion protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SNAP, soluble NSF attachment protein; SNAP-23, 23-kDa isoform of SNAP-25; SNAP-25, synaptosome-associated protein of 25 kDa; hSNAP-23 and mSNAP-23, human and murine SNAP-23, respectively; SNARE, soluble NSF attachment protein receptor; VAMP, vesicle-associated membrane protein.

Montecucco and Schiavo, 1994; Niemann et al., 1994), and BoNT/C proteolyzes syntaxin (Blasi et al., 1993b; Schiavo et al., 1995) and SNAP-25 (Foran et al., 1996; Williamson et al., 1996).

When docked synaptic vesicles fuse at release sites of the nerve terminal with the presynaptic membrane, the three substrates syntaxin, SNAP-25, and VAMP/synaptobrevin are thought to associate into low-energy ternary complexes involving subdomains with pronounced heptad symmetry of hydrophobic residues (Hayashi et al., 1994). The energy released during formation of the complex is thought to drive the actual fusion reaction (Jahn and Hanson, 1998; Weber et al., 1998). Related complexes, composed of isoforms of the three synaptic proteins, are crucial for the fusion of transport vesicles in nonneuronal cells (Ferro-Novick and Jahn, 1994; Rothman, 1994). Some of these vesicle-mediated transport steps can be blocked through the action of clostridial neurotoxins (Galli et al., 1994; Ikonen et al., 1995).

The L chains of clostridial neurotoxins form a class of proteases with a unique substrate specificity. They cleave only one of several identical peptide bonds in their respective target molecules and fail to proteolyze short peptides spanning the individual cleavage regions (Shone et al., 1993; Yamasaki et al., 1994a). Based on these observations, it was proposed that the proteases detect specific sequence motifs present in all target molecules. Searches for such common structural features in the three substrates led Rossetto and colleagues (1994) to suggest that a helical substrate recognition motif [soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) motif] present in multiple copies in synaptobrevin, syntaxin, and SNAP-25 mediates specific recognition by clostridial neurotoxins. Studies using mutational analysis in synaptobrevin demonstrated that these motifs are involved in target recognition. These studies also indicated that all five synaptobrevin-specific neurotoxins recognize the target in distinct ways (Pellizari et al., 1996, 1997). Recently, Washbourne et al. (1997) stated that efficient cleavage of SNAP-25 by BoNT/A and E also required the presence of at least one copy of such common recognition motifs.

In this study, we investigated the molecular basis of interaction between BoNT/A, C, and E with SNAP-25 and two nonneuronal SNAP-25 isoforms, human SNAP-23 (hSNAP-23) (Ravichandran et al., 1996) and mouse SNAP-23 (mSNAP-23) (also termed synet; Araki et al., 1997; Wang et al., 1997). By using a proteolytically inactive BoNT/A L chain, we established, for the first time, a toxin L chain/substrate binding assay. Specific deletion and point mutants were generated from SNAP-25 and its isoforms to analyze the contribution of subdomains or individual residues to binding and proteolysis. The data are compatible with a substrate-induced conformational fit. Furthermore, we show that single amino acid replacements generate hSNAP-23 derivatives that can be cleaved by BoNT/A and E.

## MATERIALS AND METHODS

### Plasmid constructions

DNA fragments encoding individual L chains were obtained by PCR, using purified bacteriophage-specific (BoNT/C) or total bacterial DNA [BoNT/A (strain 62A) and E (Beluga)], respectively, and were cloned in pQE3 (Qiagen, Hilden, Germany). Mutants of SNAP-25- and hSNAP-23-specific plasmids were constructed by PCR in pGEX-KG (Guan and Dixon, 1991), pGEX-2T (Pharmacia, Freiburg, Germany), pQE3, or pSP72 (Promega, Heidelberg, Germany).

### Expression and purification of recombinant proteins

The *E. coli* strain M15pREP4 (Qiagen) was transfected with the individual L chain-encoding plasmids, and proteins were produced and purified according to the manufacturer's instructions except that BoNT/C and BoNT/E L chains were induced for 3 h of incubation at 21 or 30°C, respectively. Fractions containing the L chains were dialyzed against toxin assay buffer (150 mM K glutamate, 10 mM HEPES-KOH, pH 7.2), frozen in liquid nitrogen, and kept at -70°C.

Glutathione-S-transferase (GST), GST-SNAP-25 variants, GST-syntaxin 1a, and GST-synaptobrevin 2 were affinity purified on glutathione (GT)-Sepharose (Pharmacia) according to Guan and Dixon (1991) and finally dialyzed against toxin assay buffer.

### In vitro transcription and translation

BoNT/A L chain (Glu<sup>224</sup>Gln)-, SNAP-25-, hSNAP-23-, or mSNAP-23-specific mRNAs were synthesized in vitro from suitable transcription plasmids (Binz et al., 1994; Wang et al., 1997) linearized downstream from the coding regions. Translations were performed in reticulocyte lysate (Promega), using 0.25 μg mRNA in the presence of [<sup>35</sup>S]methionine (24 μCi, 1,200 Ci/mmol; ICN Biomedicals, Irvine, CA, U.S.A.) in a total volume of 25 μl.

### Toxin treatment

A cleavage assay contained 1 μl of the translation mixture of [<sup>35</sup>S]methionine-labeled variants of SNAP-25, hSNAP-23, or mSNAP-23 and the respective L chain and was incubated for 60 min at 37°C in a total volume of 10 μl of toxin assay buffer. *E. coli* expressed GST-SNAP-25-His<sub>6</sub> variants (3 μM final concentrations) were incubated in a total volume of 100 μl of toxin assay buffer containing L chains. Aliquots (15 μl) were withdrawn at specified time intervals. Reactions were stopped by the addition of 15 μl of double-concentrated sample buffer [120 mM Tris-HCl, pH 6.75, 10% (vol/vol) β-mercaptoethanol, 4% (wt/vol) sodium dodecyl sulfate (SDS), 20% (wt/vol) glycerol, 0.014% (wt/vol) bromophenol blue]. Samples were boiled for 3 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970), using 12.5 or 15% gels. Proteins were visualized by staining with Coomassie Blue or by fluorography and quantified with a Sharp JX-325 high-resolution scanner by using an ImageMaster TM 1-D program (version 1.10; Pharmacia). Alternatively, radiolabeled samples were analyzed with a BAS-1500 phosphoimager (Fuji Photo Film, Japan).

For determination of the BoNT/C cleavage site in SNAP-25, 32 μg of SNAP-25-His<sub>6</sub> was incubated for 16 h at 37°C with recombinant L chain (250 nM final concentration) in a total volume of 100 μl of toxin assay buffer. Cleavage products were isolated by reversed-phase chromatography, using a Nucleosil 5-mm C8 column (250 × 4 mm) from Macherey and Nagel (Düren, Germany). N-terminal amino acid sequences were de-

termined on a Model 473A protein sequencer from Applied Biosystems (Foster City, CA, U.S.A.).

#### Toxin binding assay

Various GST-fusion proteins (0.1 nmol each) prebound to 10  $\mu$ l of GT-Sepharose beads were suspended in 190  $\mu$ l of toxin assay buffer containing 0.02% Triton X-100. Beads were then incubated for 30 min at 4°C with identical amounts of radio-labeled BoNT/A (Glu<sup>224</sup>Gln) L chain, as generated by *in vitro* transcription/translation. The beads were collected by centrifugation. Unbound material was recovered from the supernatant by trichloroacetic acid precipitation. Beads were washed three times each with 50 bed volumes of the same buffer. The washed pellet fraction was boiled in SDS sample buffer and analyzed together with the supernatant fraction by SDS-PAGE and fluorography.

For determination of the BoNT/A-SNAP-25 dissociation constant ( $K_D$ ), GST-SNAP-25 was incubated in the presence of various concentrations of BoNT/A (Glu<sup>224</sup>Gln) L chain ranging from 156 to 2,500 nM. The amount of bound L chain was quantified by laser densitometric scanning after SDS-PAGE and Coomassie Blue staining and corrected for nonspecific binding by subtracting the value obtained for GST. The  $K_D$  value was calculated by Scatchard plot analysis.

## RESULTS

#### Identification of the BoNT/C scissile bond in SNAP-25

Foran et al. (1996) showed that BoNT/C, in addition to proteolyzing syntaxin, also cleaves SNAP-25 in a position similar to that of BoNT/A. The precise site of proteolysis, however, was not determined. To identify the scissile bond, recombinant SNAP-25-His<sub>6</sub> was digested with recombinant L chains of BoNT/C and, as a control, BoNT/A. The products of the two reactions were analyzed by reversed-phase HPLC, and material underlying individual peak fractions was subjected to amino acid sequencing (Fig. 1). In agreement with previous results (Binz et al., 1994), the first peak fraction of the BoNT/A reaction yielded the sequence RAKMLGS-GVP. The corresponding fragment obtained in the BoNT/C reaction eluted slightly faster than the BoNT/A product and gave the sequence ATKMLGSGVP (Fig. 1, bottom panel). In both instances, material eluting after 29 min carried the authentic N-terminus of SNAP-25. We conclude that BoNT/C hydrolyzes the Arg<sup>198</sup>-Ala<sup>199</sup> bond and thus cleaves in a novel position in comparison with BoNT/A or E.

#### Mutational analyses of the BoNT/A, C, and E scissile bonds

The amino acid positions around the cleavage site of proteases are designated -P<sub>3</sub>-P<sub>2</sub>-P<sub>1</sub>-P<sub>1'</sub>-P<sub>2'</sub>-P<sub>3'</sub>-, where P<sub>1</sub>-P<sub>1'</sub> represents the actual site of proteolysis. The determination of the BoNT/C cleavage site in SNAP-25 completes the list of susceptible peptide bonds that may be cleaved by clostridial neurotoxin proteases. A comparison of these sites indicates the great diversity of the residues tolerated by this class of metalloproteases. To gain additional insights into the particular requirements for BoNT/A and E, we replaced the residues of the P<sub>1</sub>

and P<sub>1'</sub> positions by hydrophobic, other polar, negatively charged, or positively charged residues and analyzed the susceptibility of the resulting mutants (Table 1). Of five substitutions introduced at the P<sub>1</sub> position of the BoNT/A cleavage site, only threonine caused a slight but reproducible reduction of cleavage. Next, Arg<sup>198</sup> of the P<sub>1'</sub> position was systematically mutated to residues of different character. Except for a change to tyrosine, all other mutations, even the conservative exchange to lysine, dramatically reduced the sensitivity of SNAP-25 to BoNT/A (Table 1A). Similar results were obtained with BoNT/E cleavage site mutants (Table 1B). Again, substitutions in the P<sub>1'</sub> position impaired cleavage. We conclude that in accordance with previous studies regarding the BoNT/A and BoNT/B cleavage sites (Shone and Roberts, 1994; Schmidt and Bostian, 1997), the properties of the P<sub>1</sub> residue appear less critical for cleavage, whereas specific amino acid residues must be present in the P<sub>1'</sub> positions. In this respect, BoNT/C differs from the other neurotoxins: five of the seven substitutions introduced into the P<sub>1</sub> position affected proteolysis drastically (Table 1A).

#### Murine and human SNAP-25 isoforms display distinct sensitivities to cleavage by botulinal L chains

As revealed by our own and previously published data, however, proteolytic susceptibility or resistance of a potential substrate against neurotoxin proteases cannot be explained solely on the basis of residues present in the scissile bond. Recently, two isoforms of SNAP-25 were identified in human B lymphocytes (hSNAP-23; Ravichandran et al., 1996) and in murine 3T3-L1 adipocytes (mSNAP-23, also termed syndet; Araki et al., 1997; Wang et al., 1997). These nonneuronal isoforms and SNAP-25 share significant sequence identity and the amino acid residues present in the potential scissile bonds are largely conserved (Fig. 2A). It has been reported that hSNAP-23 was resistant to the action of BoNT/E (Macaulay et al., 1997). In a similar manner, mSNAP-23 was shown to resist moderate concentrations of BoNT/A and C (Chen et al., 1997) or BoNT/E (Macaulay et al., 1997). In contrast, Washbourne et al. (1997) reported that mSNAP-23 was cleaved by BoNT/E. To reexamine this issue, we incubated *in vitro* translated hSNAP-23 and mSNAP-23 with recombinant L chains of BoNT/A, C, and E (Fig. 2B). hSNAP-23 was not proteolyzed by any of the three L chains even when concentrations of up to 5  $\mu$ M were applied. In contrast, mSNAP-23 was cleaved by BoNT/E and, much less efficiently, by BoNT/A. Demonstration of successful cleavage required, however, prolonged incubation times and elevated toxin concentrations.

It remains to be shown, therefore, whether proteolysis of mSNAP-23 by BoNT/A and E would be detectable *in vivo*. In this context, it is noteworthy that the L chains of clostridial neurotoxins display a remarkable stability of several days in injected cells (Erdal et al., 1995) and, therefore, detrimental long-term *in vivo* effects of the L chains cannot

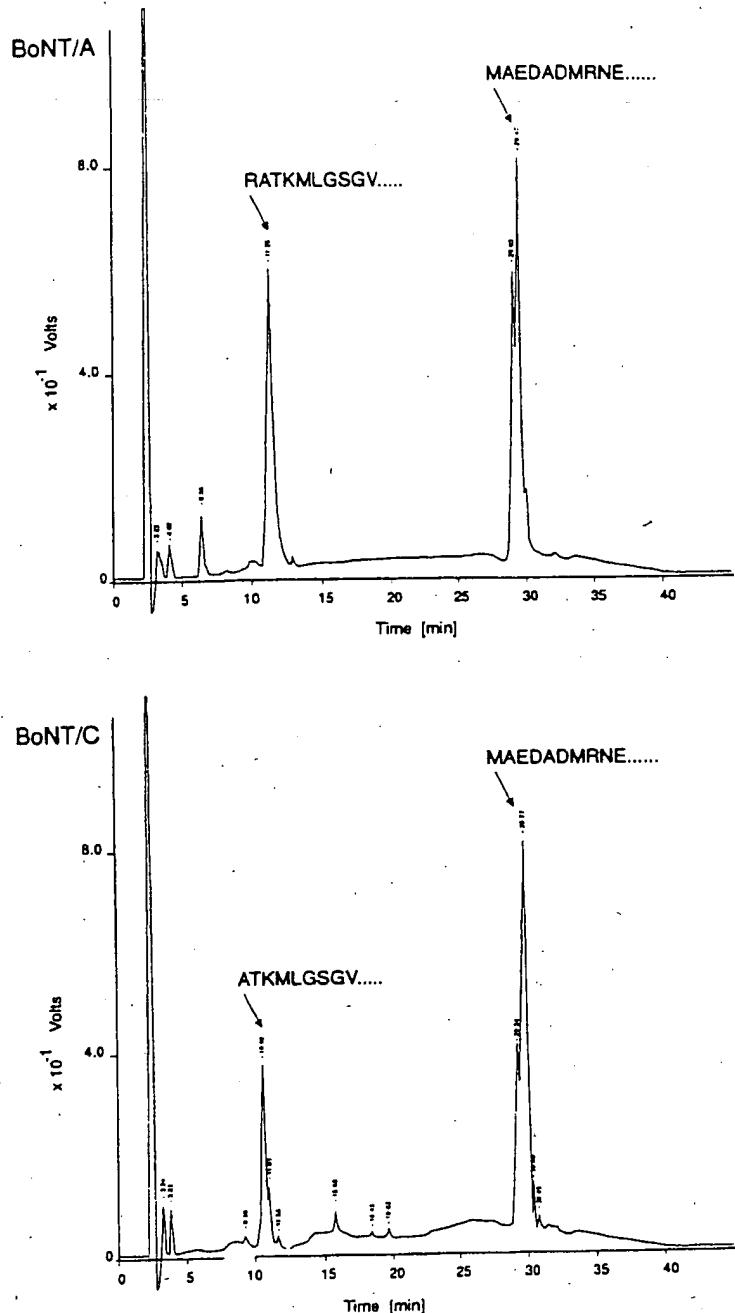


FIG. 1. Identification of the BoNT/C scissile bond in SNAP-25. Aliquots of HPLC-purified SNAP-25-His<sub>6</sub> (32  $\mu$ g each) were incubated for 16 h at 37°C with BoNT/C or BoNT/A L chains (each 250 nM final concentration). Samples were analyzed by reversed-phase HPLC using a linear gradient of 10–90% acetonitrile in 0.1% aqueous trifluoroacetic acid starting after 5 min. Amino acid sequences specify the N-terminal sequences of peptides underlying individual peak fractions.

be excluded. Furthermore, BoNT/C was reported to be almost as active as BoNT/A in mouse spinal cord cell cultures (Williamson et al., 1996), whereas it was  $\sim$ 1,000-fold less active *in vitro*. In conclusion, it is rather speculative to extrapolate from the *in vitro* cleavage rates on the *in vivo* activities of particular L chains.

#### Effects of N- and C-terminal deletions of SNAP-25 on cleavage by BoNT/A, C, and E

To define those subdomains of SNAP-25 in which individual amino acid substitutions could alter the susceptibility toward the three proteases, we next generated

a set of N- and C-terminally truncated substrate molecules. The results are presented in Fig. 3. SNAP-25(93–206) was as susceptible to cleavage by all three toxins as full-length SNAP-25. BoNT/A and E proteolyzed GST-SNAP-25(146–206) as efficiently as SNAP-25(1–206), whereas BoNT/C proteolysis was significantly decreased. The removal of additional 10 N-terminal amino acids [to yield mutant SNAP-25(156–206)] diminished cleavage by both BoNT/A and E, whereas cleavage by BoNT/C remained unaltered. SNAP-25(167–206) showed a further reduction in BoNT/C cleavage and a

TABLE 1. *Mutational analysis of the BoNT/A, C, and E cleavage sites in SNAP-25*

A.	Mutant	BoNT/A-(nM)	BoNT/C. (μM)
	BoNT/A BoNT/C		
	↓↓		
E <sup>194</sup> A N Q R A T K <sup>201</sup>		0.2	0.5
M <sup>197</sup>		0.2	Not tested
S <sup>197</sup>		0.2	Not tested
T <sup>197</sup>		0.3	Not tested
E <sup>197</sup>		0.2	Not tested
K <sup>197</sup>		0.2	Not tested
Y <sup>198</sup>		0.6	0.5
A <sup>198</sup>		60	5.3
S <sup>198</sup>		100	1.8
T <sup>198</sup>		2,500	>6.0
D <sup>198</sup>		8,000	>6.0
E <sup>198</sup>	No cleavage		>6.0
K <sup>198</sup>		100	0.5
B.	Mutant	BoNT/E (nM)	
	BoNT/E		
	↓		
Q <sup>177</sup> I D R I M E K <sup>184</sup>		0.2	
V <sup>181</sup>		0.5	
F <sup>181</sup>		100	
G <sup>181</sup>		25	
A <sup>181</sup>		2.5	
S <sup>181</sup>		10	
N <sup>181</sup>		60	

Radiolabeled substrates were generated by *in vitro* transcription/translation and incubated with various concentrations of BoNT/A, C, or E L chain in toxin assay buffer. After 1 h of incubation at 37°C, samples were analyzed by SDS-PAGE and fluorography. Percentage of cleavage was quantified by laser densitometric scanning. Data are mean values of three independent experiments and represent the concentration required to reach 50% proteolysis. The amino acid single letter code was used.

drastic reduction in BoNT/E cleavage, and BoNT/A proteolysis was no longer detectable. However, application of a higher BoNT/A L chain concentration (100 nM final concentration) resulted in 50% cleavage of this mutant after 60 min (data not shown). It is unlikely that the increased resistance of truncated SNAP-25 derivatives is merely due to steric hindrance imposed by the presence of the N-terminally located GST, as such hindrance should affect the three L chains to the same extent.

In parallel experiments we examined the impact of C-terminal deletions of SNAP-25 on cleavage by the three L chains. SNAP-25 derivatives retaining as few as five, four, and six residues on the C-terminal side of the respective scissile bonds, were cleaved by BoNT/A, C, and E as efficiently as full-length SNAP-25 (data not shown).

Together, these data indicate that interaction of BoNT/A, C, and E with SNAP-25 depends on distinct domains; BoNT/A requires the presence of the domain Met<sup>146</sup>–Met<sup>202</sup>. For BoNT/E, the minimal segment that allows efficient cleavage encompasses the segment Ile<sup>156</sup>–Asp<sup>186</sup>, whereas BoNT/C interaction depends on a larger substrate structure involving regions Asn<sup>93</sup> to Glu<sup>145</sup> and Ile<sup>156</sup> to Met<sup>202</sup>.

Binding of the BoNT/A L chain to SNAP-25 depends on the presence of the region Met<sup>146</sup>-Gln<sup>197</sup>

Before cleavage, toxin and substrate must form an enzyme-substrate complex involving contacts at one or multiple regions in both molecules. We investigated, therefore, whether it was possible to dissect the overall cleavage process into individual substeps involving assembly into the enzyme-substrate complex and a subsequent cleavage step. To prevent proteolysis of individual SNAP-25 derivatives during assembly, we replaced

A

SNAP-25	.145- E M D E N L E Q V S G I I G N L R H M A L D M G N E I D T Q N R Q I
hSNAP-23	.151- E M E E N L T Q V G S I I L G N L K D M A L N I G N E I D A Q N P Q I
mSNAP-23	.150- E M E E N L T Q V G S I I L G N L K N M A L D M G N E I D A Q N Q Q I

	BONT/E	BONT/A C
SNAP-25	179- D R I M E K A D S N K T R I D E A N O R A T K M L G S G - 206	↓ ↓
hSNAP-23	185- K R I T D K A D T N R D R I D I A N A R A K K L I D S - 211	
mSNAP-23	184- O K I T E K A D T N K N R I D A N T R A K K L I D S - 210	

B

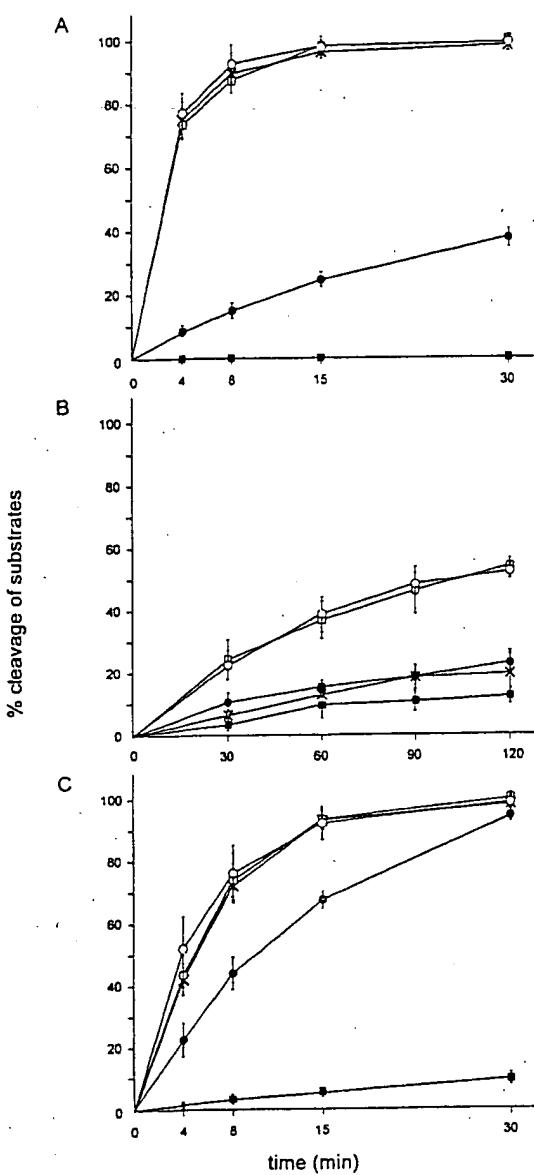


FIG. 3. Identification of the minimal essential domains of SNAP-25 required for cleavage by BoNT/A, C, and E. The cleavage rates of BoNT/A, C, and E were determined with GST-SNAP-25-His<sub>6</sub> or GST-SNAP-25 mutants, respectively, as detailed in Materials and Methods. Toxin concentrations used were 1 nM (BoNT/A; A), 500 nM (BoNT/C; B), and 1 nM (BoNT/E; C). Aliquots of the reaction mixtures were taken at the time points indicated and analyzed by 12.5% SDS-PAGE and Coomassie Blue staining. Amounts of proteins were quantified by laser densitometric scanning. Data are mean  $\pm$  SD values of four to six independent experiments. GST-SNAP-25(1-206) (□); GST-SNAP-25(93-206) (○); GST-SNAP-25(146-206) (×); GST-SNAP-25(156-206) (●); GST-SNAP-25(167-206) (■).

Glu<sup>224</sup> of the catalytic zinc-binding domain His<sup>223</sup>-Glu-Leu-Ile-His<sup>227</sup> by Gln<sup>224</sup>. As shown previously, this mutant lacks proteolytic activity (Blasi et al., 1993a), probably because the  $\delta$ -carboxyl group of Glu<sup>224</sup> is essential for stabilizing a water molecule in one of the

tetrahedral positions around the catalytic zinc ion. As the mutated protein retains the zinc ion (Yamasaki et al., 1994b), the structural changes should be minimal and the mutated L chain should be a good candidate to study its binding to GST-SNAP-25 derivatives.

Radiolabeled BoNT/A(Glu<sup>224</sup>Gln) L chain was generated by in vitro translation and was incubated with GST-SNAP-25 immobilized on GT-Sepharose beads. Bound (pellet fraction) and unbound material (supernatant fraction), were analyzed by SDS-PAGE and fluorography (Fig. 4A). The concentration of the bound GST-fusion protein was controlled by SDS-PAGE and Coomassie Blue staining (Fig. 4B). Specific binding of the BoNT/A L chain to GST-SNAP-25 was indeed observed. No binding was obtained with GT-Sepharose beads carrying GST alone, GST-syntaxin(1-267), or GST-synaptobrevin(1-116). This experimental approach was subsequently used to map those subdomains of SNAP-25 that were involved in the binding of the L chain. A deletion of the 25 N-terminal residues of SNAP-25 reduced binding of the L chain only slightly. A further deletion up to Thr<sup>46</sup> reduced binding about threefold. SNAP-25(146-206), lacking the N-terminal three-fourths of the molecule, still retained  $\sim$ 10% binding capacity of full-length SNAP-25. Complete loss of binding occurred on deletion of an additional 10 amino acids yielding SNAP-25(156-206). It is interesting that a SNAP-25 derivative containing the entire N-terminal sequence up to the BoNT/E scissile bond [SNAP-25(1-180)] failed to trap the mutant L chain, indicating that the region between the BoNT/E and BoNT/A scissile bonds is also essential for toxin binding. This is corroborated by the finding that SNAP-25(1-197), the product of the BoNT/A reaction, exhibited 65% binding capacity of full-length SNAP-25. In summary, the minimal essential fragment of SNAP-25 capable of binding to the BoNT/A L chain requires the presence of the region Met<sup>146</sup>-Gln<sup>197</sup>.

Binding experiments, using the in vitro translated radiolabeled L chain and GST-SNAP-25, provided a rapid approach for mapping the various interacting domains. A drawback of this approach, however, was the relative inefficiency of binding. Additional binding studies indicated that maximal binding of the L chain occurred within 30 min of incubation, and overnight incubations did not bring an improvement. In a separate set of experiments, we next used an inactive *E. coli*-derived BoNT/A L chain. By using this recombinant L chain, binding to SNAP-25 was saturable yielding an L chain to SNAP-25 ratio of  $\sim$ 1:1, as evidenced by densitometric scanning of Coomassie Blue-stained gels (Fig. 5A). The dissociation constant of such complexes was determined by Scatchard plot analyses yielding a  $K_D$  of  $1.9 \times 10^{-7}$  M (Fig. 5B).

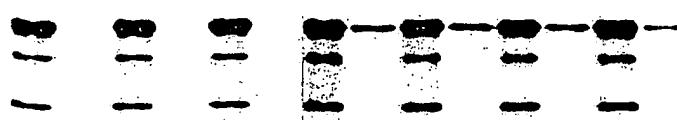
#### Mutational analysis of the region Met<sup>146</sup>-Gly<sup>155</sup>

The above binding studies showed that the best binding to BoNT/A L chain occurred already with full-length SNAP-25. Binding gradually decreased on N-terminal deletions and was no longer detectable with derivatives

FIG. 4. Binding of the BoNT/A L chain to SNAP-25 depends on the segment Met<sup>146</sup>-Gly<sup>155</sup>. A: Radiolabeled BoNT/A(Glu<sup>224</sup>Gln) L chain was incubated with GST-Sepharose beads precharged with GST, GST-syntaxin, GST-synaptobrevin, and GST-SNAP-25-His<sub>6</sub> fusion proteins. Numbers specify amino acid residues of SNAP-25. Supernatant (S) and washed pellet (P) fractions were analyzed by SDS-PAGE and fluorography. Binding (presence of radiolabeled L chain in the pellet fraction) can be demonstrated as long as the capture molecule contains the segment Met<sup>146</sup>-Gly<sup>155</sup> and the region between the BoNT/E and BoNT/A scissile bonds. B: Analyses of pellet fractions by SDS-PAGE and Coomassie Blue staining to control for the amounts of capture molecules. Lane 1, GST; lane 2, GST-syntaxin; lane 3, GST-synaptobrevin; lane 4, GST-SNAP-25(1-206)-His<sub>6</sub>; lane 5, GST-SNAP-25(14-206); lane 6, GST-SNAP-25(25-206); lane 7, GST-SNAP-25(36-206); lane 8, GST-SNAP-25(46-206); lane 9, GST-SNAP-25(93-206)-His<sub>6</sub>; lane 10, GST-SNAP-25(146-206)-His<sub>6</sub>; lane 11, GST-SNAP-25(156-206)-His<sub>6</sub>; lane 12, GST-SNAP-25(167-206)-His<sub>6</sub>; lane 13, GST-SNAP-25(1-197); lane 14, GST-SNAP-25(1-180).

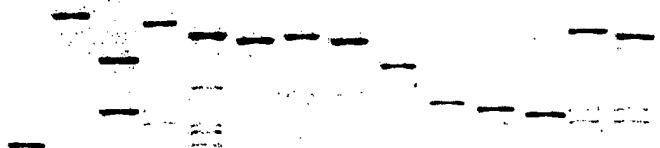
A

GST		GST - Syntaxin		GST - Synaptobr.		GST-SNAP-25					
S	P	S	P	S	P	1 - 206	14 - 206	25 - 206	36 - 206	1 - 197	1 - 180



B

1	2	3	4	5	6	7	8	9	10	11	12	13	14
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lacking the Met<sup>146</sup>-Gly<sup>155</sup> segment. Related copies of this segment have been detected in each of the three substrates and their impact on the cleavage reaction has been studied for some of the clostridial neurotoxin L chains (Rossetto et al., 1994; Pellizzari et al., 1996, 1997). To clarify the role of the Met<sup>146</sup>-Gly<sup>155</sup> segment in greater detail, we constructed mutants in which negatively charged amino acid residues were replaced by uncharged polar residues or in which the entire motif was deleted. As shown in Fig. 6, a replacement of Asp<sup>147</sup> and Glu<sup>148</sup> by Asn and Gln had only a minor impact on cleavage by BoNT/A and E. In a similar manner, a Glu<sup>151</sup>Thr replacement (as present in hSNAP-23 and mSNAP-23) had no effect on the cleavage rate (data not shown). The removal of the entire region from SNAP-25(93-206) reduced the rate of proteolysis to levels that were obtained with SNAP-25(156-206). We conclude that the presence of the region Met<sup>146</sup>-Gly<sup>155</sup> is indeed essential for efficient toxin-substrate interaction. The observation that the negatively charged amino acids may be replaced by related polar residues without changing the cleavage rate excludes the possibility that this segment interacts with the BoNT/A L chain via ionic forces.

It remains to be shown whether the polar residues are in fact engaged in a direct interaction with the L chain or whether they are part of a sensor that controls folding or the substrate into a particular secondary structure.

#### Single amino acid replacements in hSNAP-23 confer susceptibility to BoNT/A and E

The SNAP-25 segment Met<sup>146</sup>-Gln<sup>197</sup> represents a minimal length substrate for both BoNT/A and E. Only a limited number of amino acid changes are found in the corresponding regions of hSNAP-23 and mSNAP-23 (Fig. 2A). Such residues could mark direct interaction sites with the toxin L chains. Alternatively, they could have a profound influence on the secondary structure of the substrate and could, therefore, influence the toxin target interaction indirectly. To analyze the role of particular residues in greater detail, we replaced some of them in hSNAP-23 by those found in rat SNAP-25.

A Thr<sup>158</sup>Glu mutation yielded a product that remained resistant to all three botulinal L chains (Table 2). In SNAP-25, an aspartate residue is found in the P<sub>2</sub> position of the BoNT/E cleavage site, whereas the poorly susceptible mSNAP-23 carries a glutamine and the toxin-resis-

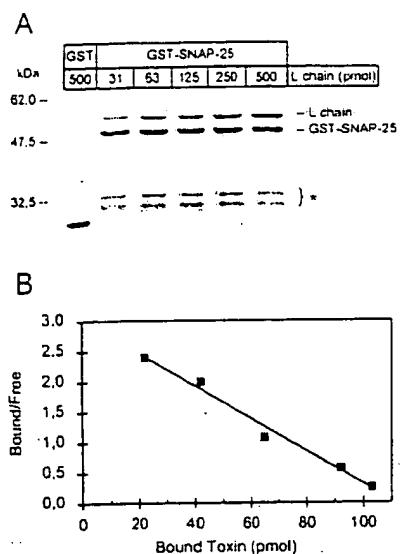


FIG. 5. Scatchard plot analysis of BoNT/A L chain binding to SNAP-25. A: GST or GST-SNAP-25 immobilized on GT-Sepharose beads was incubated with the indicated amounts of recombinant BoNT/A(Glu<sup>224</sup>Gln) L chain. Bound L chain was determined as above by SDS-PAGE and quantified by laser densitometric scanning. Binding to SNAP-25 was saturable, yielding an L chain to SNAP-25 ratio of ~1:1. Bands labeled by the asterisk are N-terminal degradation products of GST-SNAP-25. B: The molar ratio of bound to free L chain was plotted versus the amount of bound L chain. Data are mean values of three independent experiments. The dissociation constant  $K_D$  was  $1.9 \times 10^{-7} M$ .

tant hSNAP-23 contains a lysine in this position (Fig. 2A). As indicated in Table 2, a Lys<sup>185</sup>Asp replacement in hSNAP-23 had no effect on the reactions with BoNT/A or C but generated an effectively cleaved substrate for BoNT/E. Indeed, hSNAP-23(Lys<sup>185</sup>Asp) proved to be a better substrate for BoNT/E than mSNAP-23 (Table 2). This finding is in support of a previous study, in which mutation of the P<sub>2</sub> position of the BoNT/A cleavage site in SNAP-25 drastically reduced the cleavage rate (Schmidt and Bostian, 1997). Next, we assessed the impact of an Ile<sup>173</sup>Met substitution. This residue was chosen for mutagenesis, because a similar Ile<sup>46</sup>Met replacement in rat synaptobrevin 1 dramatically increased cleavage by BoNT/D (Yamasaki et al., 1994a). Furthermore, Ile<sup>173</sup> and Ile<sup>46</sup> of hSNAP-23 and rat synaptobrevin 1, respectively, reside in identical positions with respect to the corresponding cleavage sites for BoNT/E and D. This mutant remained resistant against BoNT/E but, interestingly, showed an increased sensitivity toward BoNT/A. A Pro<sup>182</sup>Arg mutation yielded a hSNAP-23 derivative that, in contrast to wild-type hSNAP-23, could be cleaved by BoNT/A and E. Likewise, introduction of a proline residue into the equivalent position of SNAP-25 [SNAP-25(Arg<sup>176</sup>Pro)] drastically reduced cleavage by BoNT/A and E (Table 2). Together, these data demonstrate that the presence of a proline residue within the minimal essential interactive domain deteriorates the substrate quality of SNAP-25 derivatives.

## DISCUSSION

We analyzed structural features of SNAP-25 and of its nonneuronal murine and human SNAP-23 isoforms that influence proteolytic cleavage by botulinum neurotoxin L chains of serotypes A, C, and E. The following several discoveries were made that may provide additional insights into the complex mechanism of the unique sub-

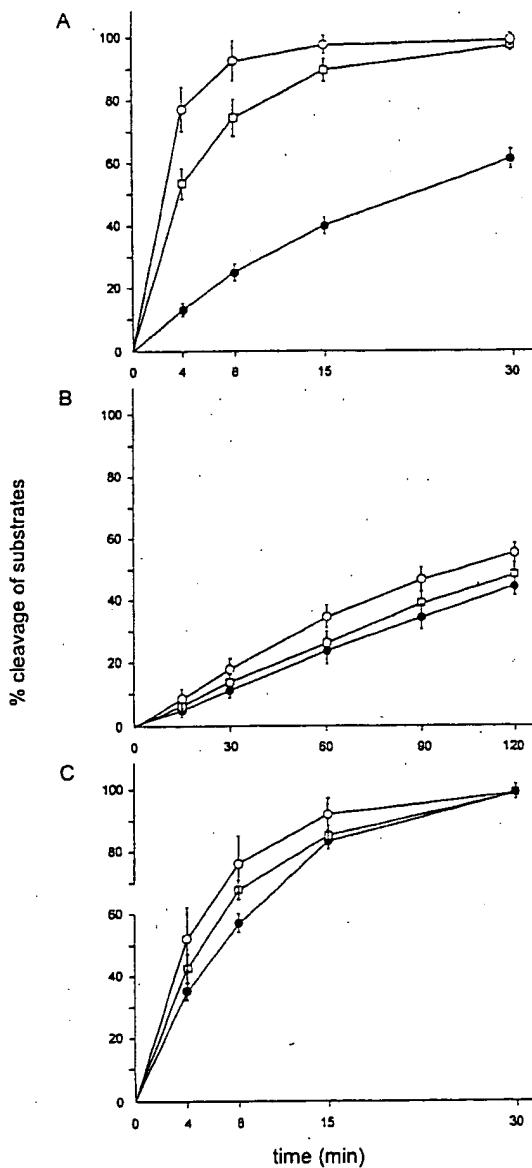


FIG. 6. Charged residues of the segment Met<sup>146</sup>-Gly<sup>155</sup> have no impact on cleavage. The cleavage rates of BoNT/A, C, and E were determined with GST-SNAP-25(93-206) as a control and several variants thereof containing mutations in the SNARE motif as detailed in Materials and Methods. Toxin concentrations used were *in vivo* (BoNT/A, A), 300 nM (BoNT/C, B), and 1 nM (BoNT/E, C). Aliquots of the individual reaction mixtures were taken at the time points indicated and analyzed as in Fig. 3. GST-SNAP-25(93-206) (○); GST-SNAP-25(93-206)Asp<sup>147</sup>Asn, Glu<sup>148</sup>Gln (□); GST-SNAP-25(93-206)Δ145-153 (○).

TABLE 2. Effect of point mutations in SNAP-25 and hSNAP-23 on cleavage with BoNT/A, C, and E

Mutant	BoNT/A	BoNT/C	BoNT/E
SNAP-25	49.7 ± 8.0	36.1 ± 6.0	48.8 ± 6.5
SNAP-25 (Arg <sup>176</sup> Pro)	24.0 ± 5.5	39.8 ± 6.6	15.4 ± 3.2
hSNAP-23	No cleavage	No cleavage	No cleavage
hSNAP-23 (Thr <sup>158</sup> Glu)	No cleavage	No cleavage	No cleavage
hSNAP-23 (Ile <sup>173</sup> Met)	0.9 ± 0.2	No cleavage	No cleavage
hSNAP-23 (Pro <sup>182</sup> Arg)	7.0 ± 1.0	No cleavage	12.7 ± 0.6
hSNAP-23 (Lys <sup>185</sup> Asp)	No cleavage	No cleavage	100 ± 2.2
mSNAP-23	3.6 ± 0.8	No cleavage	100 ± 2.2
			38.2 ± 5.0 <sup>a</sup>

Radiolabeled substrates were generated by in vitro transcription/translation. SNAP-25 and its mutant were treated with BoNT/A and E (each at 0.3 nM final concentration) and BoNT/C (0.5 μM). For mSNAP-23, hSNAP-23, and its mutants, toxin L chains were applied at 1 μM final concentration.

<sup>a</sup> Experiments done with 125 nM final concentration of BoNT/E L chain. All incubations were performed for 60 min at 37°C and cleavage was analyzed by SDS-PAGE and fluorography. Data represent mean ± SD values of four independent experiments and are expressed as percentages of cleavage.

strate recognition by clostridial L chain proteases: (1) The three proteases cleave the identical substrate. Each of them, however, shows distinct requirements regarding the minimal essential domains for cleavage. (2) The L chains of BoNT/A and E require specific residues around the scissile bond in the P<sub>2</sub> and P<sub>1</sub>' positions, whereas residues in the P<sub>1</sub> position are less crucial. For BoNT/C, the nature of the residue in the P<sub>1</sub> position is also important. (3) Using a proteolytically inactive BoNT/A L chain, we developed an in vitro binding assay that allowed us to distinguish substrate domains that are important for enzyme–substrate complex formation and for subsequent proteolysis. Our studies indicate that the actual cleavage reaction constitutes the rate-limiting step. The minimal essential domains required for optimal cleavage of SNAP-25 by BoNT/A, C, and E extend from Met<sup>146</sup> to Met<sup>202</sup>, Asn<sup>93</sup> to Met<sup>202</sup>, and Met<sup>146</sup> to Asp<sup>186</sup>, respectively. (4) Within these domains, single amino acid substitutions may drastically alter the susceptibility toward individual L chains. The closely related nonneuronal isoform mSNAP-23 is cleaved by BoNT/E and by high concentrations of BoNT/A but not by BoNT/C. In contrast, hSNAP-23 is resistant against all three L chains. Single amino acid substitutions partially restore cleavage of hSNAP-23. (5) Together, our findings are compatible with the possibility that the clostridial L chains turn into active proteases only on contact with their cognate substrate.

Before we discuss the impact of SNAP-25 subdomains on binding and cleavage in detail, we should recall that in the cell the SNARE proteins SNAP-25, synaptobrevin, and syntaxin exist in at least two dis-

tinct conformational states, only one of which allows proteolytic degradation by clostridial neurotoxin L chains. The individual monomeric SNAREs have little secondary structure under physiological conditions. This follows from both NMR analyses of a synthetic synaptobrevin polypeptide (Cornille et al., 1994) and circular dichroic spectroscopy of SNAP-25 (Fasshauer et al., 1997). These unprotected SNAREs can be attacked by clostridial L chains (Hayashi et al., 1994). On contact with other SNARE proteins, synaptobrevin, SNAP-25, and syntaxin assemble spontaneously into various low-energy heterodimeric or heterotrimeric complexes. Formation of such complexes is driven by coiled-coil formation (Hayashi et al., 1994; Fasshauer et al., 1997) and paralleled by the gain of resistance against clostridial neurotoxins (Hayashi et al., 1994). Such complexes may be dissociated at the expense of ATP through the joint action of α-SNAP [α-soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein] and NSF yielding again toxin-sensitive SNARE monomers (Hayashi et al., 1995). According to our current view of membrane fusion (Jahn and Hanson, 1998; Weber et al., 1998), the conformational energy stored in the unfolded SNARE monomers is liberated during assembly into SNARE complexes and used to pull the vesicle and target membranes toward each other. On contact with a neurotoxin L chain, however, such conformational energy could also drive conformational changes in either the substrate alone, or, perhaps more likely, in both the substrate and the L chain, resulting in the formation of stable enzyme–substrate complexes.

Within the minimal interactive domains of SNAP-25 determined for BoNT/A, C, and E, the individual cleavage site region, encompassing the scissile bond itself and ~20 residues located N-terminally thereof, constitutes a most critical subdomain influencing both substrate binding and cleavage. As demonstrated here with a proteolytically inactive BoNT/A L chain, this region clearly contributes to binding, as the BoNT/A cleavage product (ending with Gln<sup>197</sup>) retains 65% binding activity of full-length SNAP-25 and the BoNT/E product (ending with Arg<sup>180</sup>) fails to bind. Efficient binding, however, does not necessarily imply efficient cleavage. This follows from the observation that the SNAP-25(Arg<sup>198</sup>Glu) mutant binds to the BoNT/A L chain with a similar efficiency as wild-type SNAP-25 (T. Binz, unpublished observation) without being subsequently cleaved (Table 1). Our mutational analyses of the cleavage sites of BoNT/A and E further support the theory that the nature and the size of residues present in the P<sub>1</sub>' and in the P<sub>2</sub> position are crucial for cleavage, whereas those in the P<sub>1</sub> position are not. Similar findings have been reported by Schmidt and Bostian (1997) who showed, in addition, that residues of the P<sub>3</sub>' and P<sub>4</sub> positions have little impact on cleavage. BoNT/C differs from the other two proteases, as changes in P<sub>1</sub> influence cleavage drastically (Table 1).

Comparative binding and cleavage analyses of N-terminal deletion mutants of SNAP-25 brought the surprising insight that BoNT/A required the entire SNAP-25 molecule for optimal binding, whereas cleavage was not affected unless these deletions affected the minimal size substrate represented by SNAP-25(Met<sup>146</sup>-Met<sup>202</sup>). Binding to full-length SNAP-25 was saturable, yielding a 1:1 ratio and a  $K_D$  of  $1.9 \times 10^{-7} M$ . Even a deletion of the 13 N-terminal residues of SNAP-25, however, reduced binding already to ~60% of full-length SNAP-25. In comparison, binding to SNAP-25(Met<sup>146</sup>-Met<sup>202</sup>) was ~10% without affecting the rate of proteolysis. These findings suggest that substrate recognition and binding occur faster than the actual cleavage reaction. A deletion of an additional 10 residues, to yield SNAP-25(Ile<sup>156</sup>-Gly<sup>206</sup>), reduced binding below detectable levels and drastically reduced cleavage by BoNT/A, but much less dramatically by BoNT/E. For BoNT/C, a more extended substrate structure, encompassing residues Asn<sup>93</sup> to Met<sup>202</sup> was required to establish optimal cleavage. It is surprising that a deletion of the segment Glu<sup>145</sup> to Ser<sup>154</sup> from this substrate affected cleavage only slightly.

What is the function of the segment Glu<sup>145</sup> to Ser<sup>154</sup> and of related motifs in the other SNAREs? These motifs are highly conserved during evolution, suggesting that they could serve a common, as yet unknown, function in the individual SNAREs. Recently, Washbourne et al. (1997) reported that a deletion of the entire N-terminal domain including the Glu<sup>145</sup>-Ser<sup>154</sup> domain resulted in a dramatic loss of cleavage by reduced BoNT/A and E holotoxins. The authors concluded that this segment was indeed essential for toxin action. These findings agree with our observation that cleavage by BoNT/A and E was optimal only with substrates containing this segment. In our study, however, effective cleavage by the recombinant BoNT/E L chain continued also with substrates lacking this segment. The reason for this discrepancy is unclear. One approach to clarify the role of this segment could involve studies on potentially inhibitory properties of synthetic peptides representing this Glu<sup>145</sup>-Ser<sup>154</sup> segment. Unfortunately, a corresponding peptide was found to be insoluble (Rossetto et al., 1994). Studies with related segments in synaptobrevin had demonstrated that the presence of the negatively charged residues was obligatory for cleavage (Pellizzari et al., 1996, 1997). To our surprise, however, substitutions of these acidic amino acid residues in SNAP-25 by the corresponding noncharged amides affected cleavage only slightly. Considering that BoNT/A, C, and E share the property of tolerating the uncharged amino acids with BoNT/D (Pellizzari et al., 1997), we may conclude that the clostridial neurotoxins are specialized proteases that have evolved from one ancestral gene, recognizing originally the same repetitive motif in their individual substrates. During evolution, however, each neurotoxin may have developed its individual dependency on particular residues within this motif. Following these lines of arguments, BoNT/E and C show the least dependency; substrates lacking the Glu<sup>145</sup>-Ser<sup>154</sup> motif are cleaved

only slightly worse or even equally well, respectively (Fig. 6).

At least for BoNT/A, the cleavage reaction appears to constitute the rate-limiting step. This suggests that on a first contact, SNAP-25 induces a conformational change of the BoNT/A L chain, as has been reported for the interaction between tetanus toxin and its substrate synaptobrevin (Cornille et al., 1997). This could explain why hSNAP-23 and mSNAP-23 are poor substrates. Even after mutational correction of the SNARE motifs and despite the presence of largely conserved cleavage sites, mSNAP-23 was cleaved by high concentrations of BoNT/E only and, much less efficiently, by BoNT/A, whereas hSNAP-23 remained resistant against all three L chains. In keeping with these findings, binding of mSNAP-23 to BoNT/A was barely detectable and no binding of hSNAP-23 was observed. This is remarkable, as hSNAP-23 and SNAP-25 share 63% identical residues within the domain mapped to be essential for optimal cleavage by BoNT/A. Unfortunately, circular dichroism spectroscopy of BoNT/A-SNAP-25 complexes did not reveal a change in  $\alpha$ -helicity. However, such a difference would probably not be detected, if new helices were formed at the expense of previously existing helices. An Arg<sup>176</sup>Pro substitution in SNAP-25 drastically reduced the cleavability by BoNT/A and E but not by BoNT/C. Likewise, the reverse mutation in hSNAP-23 restored cleavability by BoNT/A and E (Table 2). The simplest interpretation of these findings would imply that Arg<sup>176</sup> of SNAP-25 is required to interact with the two L chains. An alternative hypothesis would suggest that introduction of helix-breaking residues prevents formation of the Michaelis complex. Clearly, this issue demands more efforts and mutants and more sophisticated techniques such as stopped-flow analyses. At any rate, the generation of toxin-sensitive SNAP-23 mutants could provide potentially valuable tools to study the role of these SNAREs in apical transport in polarized cells (Galli et al., 1998).

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# PRODUCT APPLICATION FOCUS

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## Immunoaffinity Purification of FLAG® Epitope-Tagged Bacterial Alkaline Phosphatase Using a Novel Monoclonal Antibody and Peptide Elution

Bill L. Brizzard, Richard G. Chubet and Douglas L. Vizard  
Eastman Kodak Company, New Haven, CT, USA.

### ABSTRACT

The FLAG® epitope is an eight amino acid peptide (AspTyr-LysAspAspAspAspLys) that is useful for immunoaffinity purification of fusion proteins. A monoclonal antibody (anti-FLAG® M1) that binds the FLAG epitope in a calcium-dependent manner and requires an N-terminal FLAG sequence has been described previously. We describe the use of a second anti-FLAG monoclonal antibody (anti-FLAG M2) in immunoaffinity purification of N-terminal Met-FLAG and C-terminal FLAG fusion to bacterial alkaline phosphatase. Although binding of an anti-FLAG M2 monoclonal antibody to the FLAG epitope is not calcium-dependent, bound fusion proteins can be eluted by competition with FLAG peptide.

### INTRODUCTION

The FLAG® protein expression and purification system is based on the fusion of an eight amino acid marker peptide (FLAG) to a target protein to be expressed and purified. The FLAG sequence (AspTyrLysAspAspAspAspLys) is hydrophilic and includes the cleavage recognition sequence (AspAspAspAspLys) of the enzyme enterokinase (8). A monoclonal antibody (anti-FLAG® M1) specific for the FLAG marker fused to the N-terminus of expressed proteins has been described (14). A primary benefit of the anti-FLAG M1 monoclonal antibody is that it binds to FLAG fusion proteins in a calcium-dependent manner, allowing gentle elution by the addition of chelating agents. The FLAG system has been used for detection of proteins expressed in a variety of cell types, including examples from bacterial (2,15), yeast (8)

and mammalian (6,10,17) cells.

A limitation of the anti-FLAG M1 monoclonal antibody is that it is specific for the N-terminal FLAG fusion proteins produced following removal of a secretion signal sequence in which the first amino acid of the FLAG sequence is at the very N-terminus of the fusion protein (14). Therefore, the anti-FLAG M1 monoclonal antibody cannot be used for affinity purification of FLAG fusion proteins in which the FLAG marker is preceded by a methionine at the N-terminus of the protein (Met-FLAG fusion proteins) or the FLAG marker is fused to the C-terminus of the protein.

In an effort to extend the applications of the FLAG system, we have evaluated the potential of an additional anti-FLAG monoclonal antibody (anti-FLAG M2) for use in affinity purification of FLAG fusion proteins. The binding of the anti-FLAG M2 monoclonal antibody is not calcium-dependent, and thus bound antigens cannot be dissociated by the addition of chelating agents (unpublished observations). We have coupled the anti-FLAG M2 monoclonal antibody to agarose beads by hydrazide linkage, and this preparation has been used successfully by others for the affinity purification of N-terminal Met-FLAG fusion proteins. Initially, elution of bound proteins was carried out at low pH (11), and, more recently, synthetic FLAG peptide has been used for competitive elution (5). In this report, we describe the expression and subsequent purification by anti-FLAG M2 affinity chromatography of N-terminal Met-FLAG and C-terminal FLAG fusions to bacterial alkaline phosphatase (BAP). Furthermore, we demonstrate that synthetic FLAG peptide can be used as a gentle alternative to low pH for the elution of both N-terminal Met-FLAG and C-terminal FLAG fusion proteins.

## MATERIALS AND METHODS

### Expression Vectors and Cloning of Bacterial Alkaline Phosphatase

The pFLAG-MAC and pFLAG-CTS expression vectors (Figure 1) were derived from the pFLAG-1 expression vector (Eastman Kodak Company, New Haven, CT, USA). A clone of the *Escherichia coli* alkaline phosphatase gene, *phoA* (9), from which the leader sequence and N-terminal 4 amino acids of the mature enzyme were deleted, was kindly provided by Dr. Mark Sullivan (Kodak Research Labs, Rochester, NY, USA). The modified alkaline phosphatase gene was then subcloned into *Xba*I/*Kpn*I double-digested pFLAG-MAC to generate pFLAG-MAC-BAP and *Bgl*II/*Sal*I double-digested pFLAG-CTS to generate pFLAG-CTS-BAP (Figure 1). The nucleotide sequence of FLAG/alkaline phosphatase junctions were confirmed by DNA sequencing using the Deazaquel DNA sequencing kit (IBI/Kodak, New Haven, CT, USA). Restriction and modifying enzymes were from IBI/Kodak.

### Expression in *E. coli*

Cultures of *E. coli* LL308, *F' lacI<sup>q</sup> lacZ<sup>ΔM15</sup> lacY<sup>+</sup> pro<sup>+</sup> (Δpro-lac) thi supE recA nraA*, (12) transformed with pFLAG-MAC-BAP and pFLAG-CTS-BAP were grown overnight at 37°C in Luria broth containing 50 µg/mL ampicillin. The overnight cultures were diluted 1:100 into fresh Luria broth containing 50 µg/mL ampicillin and grown at 37°C until early log phase. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and growth was continued for an additional 2 h at 37°C.

### Preparation of *E. coli* Cell Lysate

*E. coli* LL308 cells expressing FLAG-BAP fusion protein were harvested by centrifugation; the culture broth was discarded; and the cell pellet was resuspended in Buffer A (50 mL for 500 mL of culture), 0.25 mg/mL lysozyme, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA and 50 µg/mL sodium azide. The suspension was incubated at room temperature for 10 min; Buffer B (1/10 vol) containing 1.5 M NaCl, 0.1 M CaCl<sub>2</sub>, 0.1 M MgCl<sub>2</sub>, 20 µg/mL DNase I, 150 µg/mL ovomucoid protease inhibitor (Sigma Chemical, St. Louis, MO, USA) was then added, and incubation was continued for an additional 10 min at room temperature. The lysate was then centrifuged at 25000×*g* for 1 h at 4°C. The cleared lysate was then applied to the anti-FLAG M2 affinity gel column.

### Production and Purification of Anti-FLAG M2 Monoclonal Antibody

The anti-FLAG M2 hybridoma, an NS1 myeloma BALBc/spleen cell hybrid, produces an IgG<sub>1</sub> antibody and was derived at Immunex (Seattle, WA, USA). The anti-FLAG M2 monoclonal was purified from ascitic fluid by chromatography using MAPS II Protein A Affi-Gel (Bio-Rad, Hercules, CA, USA).

### Preparation of Anti-FLAG M2 Affinity Gel

The antibody was coupled to Hydrazide AvidGel™ Ax (Bioprobe International, Tustin, CA, USA) according to the

manufacturer's instructions. Purified anti-FLAG M2 monoclonal antibody was dialyzed into coupling buffer, and the concentration of antibody was determined from the optical density at 278 nm. Following the coupling reaction, the gel was pelleted by centrifugation, and the concentration of the remaining uncoupled antibody was determined from the optical density. The amount of antibody coupled per milliliter of gel volume (typically 2.5–4.0 mg/mL) was estimated from the difference in optical density. The anti-FLAG M2 affinity gel was washed by centrifugation at 4°C in phosphate-buffered saline, pH 7.4, containing 0.02% sodium azide.

### Immunoaffinity Chromatography

Immunoaffinity chromatography of *E. coli* cell lysates containing FLAG-BAP fusion proteins was performed at room temperature. The anti-FLAG M2 affinity gel suspension was poured into a 12-mL polypropylene column to give a final bed volume of 1 mL. The column was then washed with three 5-mL aliquots of TBS (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl) followed by three 5-mL aliquots of 0.1 M glycine-HCl pH 3.5 and immediately neutralized with three 5-mL aliquots of TBS. The cleared *E. coli* LL308 lysate containing FLAG-BAP fusion protein was warmed to room temperature and applied to the column by gravity flow. The column was then washed with three 10-mL aliquots of TBS. Bound FLAG-BAP fusion protein was eluted with six 1-mL aliquots of FLAG peptide in TBS.

### Synthesis and Purification of FLAG Peptide

The FLAG peptide, NH<sub>2</sub>-AspTyrLysAspAspAspAspLys-COOH, was synthesized and purified by HPLC to a level of greater than 95% purity by the Cornell Biotechnology Analytical and Synthetic Facility (Ithaca, NY, USA). The atomic mass of the peptide as measured by mass spectral analysis was 1012.28. The molarity of the peptide in solution was calculated using the molar extinction coefficient of tyrosine in water (1420) at 274.6 nm.

### Measurement of Alkaline Phosphatase Activity and Protein Concentration

The enzymatic activity of the alkaline phosphatase enzyme was measured using an IBI Biolyzer™. Protein concentration was determined using Bradford reagent (Bio-Rad).

## RESULTS

### Expression Vectors and FLAG-BAP Gene Fusions

The pFLAG-MAC and pFLAG-CTS *E. coli* expression vectors (Figure 1A) were designed for expression of N-terminal Met-FLAG and C-terminal FLAG fusion proteins, respectively. Transcription of cloned DNA is driven by the *E. coli* *lac* promoter (1). In pFLAG-MAC, the ATG translational initiation codon is followed immediately by the 24-bp sequence encoding the eight amino acid FLAG sequence and the downstream multiple cloning site. In pFLAG-CTS, the ATG translational initiation codon is followed by the DNA

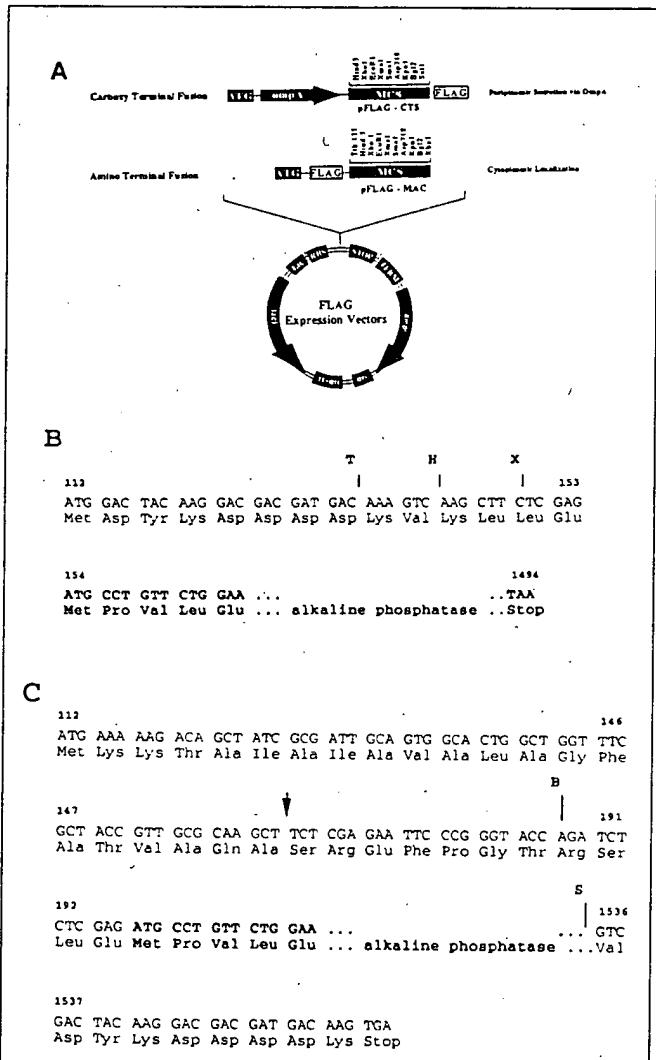
sequence encoding the *ompA* signal peptide (13) and a downstream multiple cloning site followed by the 24-DNA sequence encoding the FLAG marker.

The nucleotide and amino acid sequence of the FLAG/BAP junction in pFLAG-MAC-BAP are shown in Figure 1B. Directional cloning of the BAP gene into *Xba*I/*Kpn*I double-digested pFLAG-MAC resulted in the inclusion of an additional 15 bp of DNA sequence from the multiple cloning site between the FLAG sequence and the first base of the BAP gene. The predicted molecular weight of the Met-FLAG-BAP fusion protein is 48421 Da. The sequence of the upstream *ompA*/BAP and downstream

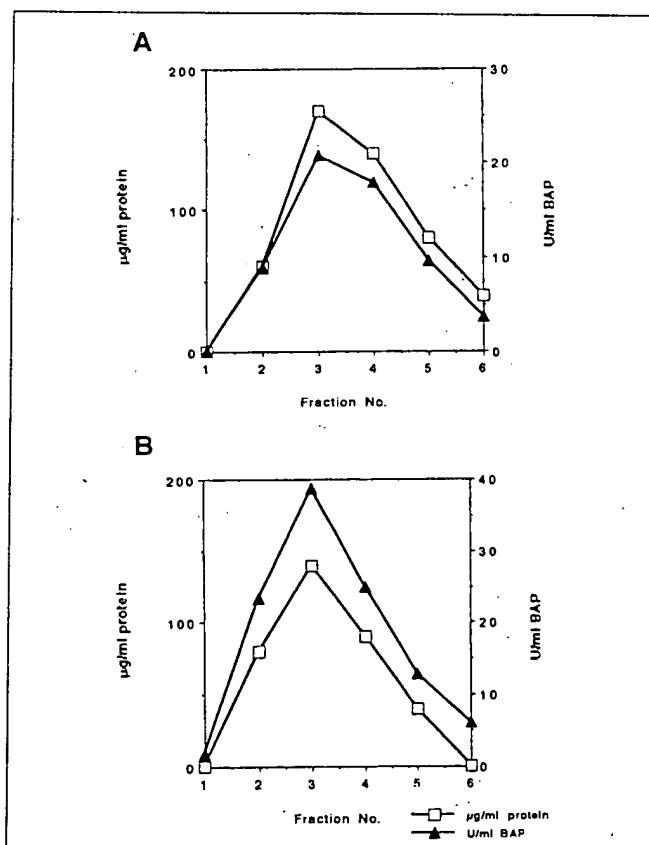
BAP/FLAG junction in pFLAG-CTS-BAP is shown in Figure 1C. Directional cloning of the alkaline phosphatase gene into *Bgl*II/*Sal*I double-digested pFLAG-CTS resulted in an additional 33 bp of sequence between the last base of the *ompA* leader peptide and the first base of the BAP gene, as well as the substitution of a valine codon for the BAP gene translational stop codon immediately preceding the C-terminal FLAG marker. The predicted molecular weight of the C-FLAG-BAP fusion protein without the *ompA* leader is 49067 Da. Because our interest was in the interaction of the anti-FLAG M2 antibody and the N-terminal Met-FLAG or the C-terminal FLAG fusion to the expressed protein, and not in the native enzyme, neither construct was further modified.

### Expression and Purification of FLAG-BAP Fusion Proteins

Cultures of *E. coli* LL308 containing pFLAG-MAC-BAP and pFLAG-CTS-BAP were induced with IPTG, and cleared lysates were prepared as described in Materials and Methods. The total protein concentration of the pFLAG-MAC-BAP and pFLAG-CTS-BAP lysates was 0.5 mg/mL and 0.3 mg/mL, respectively. The cleared lysates (40 mL of each) were applied separately to 1-mL anti-FLAG M2 affinity columns (3.8 mg anti-FLAG M2 antibody per milliliter gel). The columns



**Figure 1.** (A) pFLAG-CTS and pFLAG-MAC expression vectors. pFLAG-CTS and pFLAG-MAC are 5391 and 5056 bp, respectively. Abbreviations are: *tac* = hybrid between *trp* and *lac* promoters; RBS = ribosome binding site; *ompA* = DNA sequence encoding the leader peptide of *E. coli* outer membrane protein A; FLAG = AspTyrLysAspAspAspAspLys; MCS = multiple cloning site; STOP = DNA sequence encoding translational stop codons; TERM = *E. coli* *rrnB* transcriptional terminator; *amp*<sup>r</sup> = B-lactamase gene; ori = col E1 DNA replication origin; *f*<sub>1</sub> = *f*<sub>1</sub> phage replication origin; *lacZ* = *lacZ*<sup>+</sup> gene. (B) DNA and protein sequence of the FLAG-BAP junction in pFLAG-MAC-BAP. T, H and X represent *Tth* 111 I, *Hind* III and *Xba* I restriction enzyme cleavage sites, respectively. The sequence of the *phoA* gene is shown in bold. (C) DNA and protein sequence of the *ompA*-BAP and BAP-FLAG junctions in pFLAG-CTS-BAP. B and S represent *Bgl* II and *Sal* I restriction enzyme cleavage sites. The arrow indicates the cleavage site of *E. coli* signal peptidase. The sequence of the *phoA* gene is shown in bold.

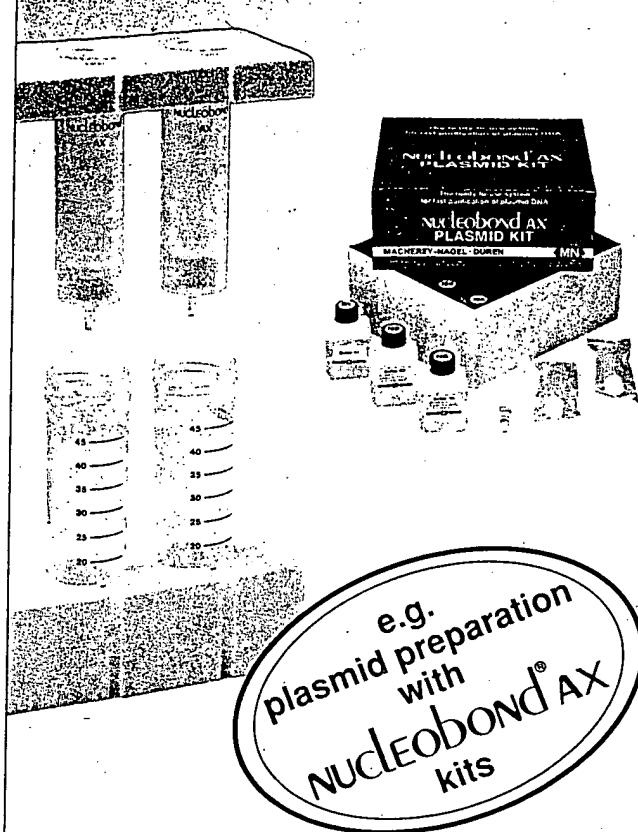


**Figure 2.** (A) Protein concentration and enzymatic activity of Met-FLAG-BAP column fractions eluted with FLAG peptide. (B) Protein concentration and enzymatic activity of C-FLAG-BAP column fractions eluted with FLAG peptide. Fractions were eluted with 0.8-molar column equivalents (fraction 1); 1.6-molar column equivalents (fraction 2); 3.2 molar column equivalents (fraction 3); 6.4-molar column equivalents (fraction 4); 8.0-molar column equivalents (fraction 5); and 16.0-molar column equivalents (fraction 6) of FLAG peptide in TBS. Alkaline phosphatase activity is given in international units (3).

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were washed with TBS, and bound FLAG-BAP fusion protein was eluted by stepwise-addition of six 1-mL aliquots of FLAG peptide at 0.8, 1.6, 3.2, 6.4, 8.0 and 16.0 molar column equivalents (1 molar column equivalent equals 50 µg FLAG peptide). The fractions were collected and analyzed for protein using Bradford reagent (the FLAG peptide is not detected by Bradford reagent) and for alkaline phosphatase activity using the Biolyzer (Figure 2A and B). Elution of FLAG-BAP fusion protein began at 1.6-molar column equivalents, and the elution peak occurred in the 3.2-molar equivalent fraction. A total of 490 µg of Met-FLAG-BAP and 350 µg of C-FLAG-BAP protein were recovered, representing 2.5% and 2.9% of the total protein applied, respectively. The specific activity of the FLAG-BAP protein in the peak fraction was 120 IU per mg for Met-FLAG-BAP and 280 IU per mg for C-FLAG-BAP.

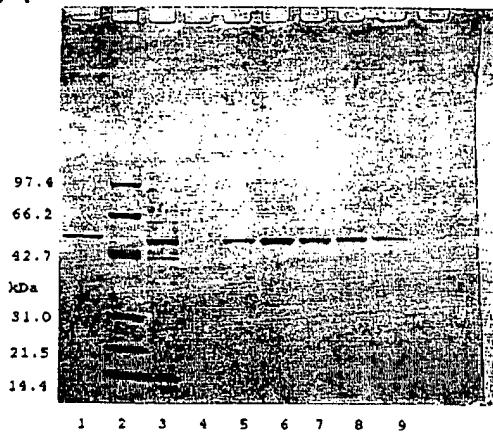
The purity of the BAP proteins eluted from each affinity column was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In each case, a single band with an electrophoretic mobility corresponding to the predicted molecular weight of the Met-FLAG-BAP (Figure 3A) and C-FLAG-BAP fusion proteins (Figure 3B) is visible in column fractions eluted with FLAG peptide.

## DISCUSSION

We have shown that N-terminal Met-FLAG-BAP and C-terminal FLAG-BAP fusion proteins can be efficiently eluted from the anti-FLAG M2 affinity column using only a several-fold molar excess of FLAG peptide. The mature alkaline phosphatase enzyme is a homodimer of two 47-kDa subunits (4). Dimerization normally occurs following secretion into the *E. coli* periplasmic space and is required for enzymatic activity (7). Alkaline phosphatase expressed in the cytoplasm is enzymatically inactive and subject to rapid proteolytic degradation. However, the purified cytoplasmic precursor will dimerize *in vitro* with slow kinetics. We found the specific activity of the purified Met-FLAG-BAP to be lower than that of the C-FLAG-BAP (Figure 2). Although not tested directly, it is possible that the lower specific activity of the purified Met-FLAG-BAP fusion protein was the result of cytoplasmic expression of the monomer followed by slow dimerization during the purification procedure.

Since enzymatically active protein was found in both the eluted N-terminal Met-FLAG-BAP and C-terminal FLAG-BAP column fractions, both preparations are likely to contain dimerized alkaline phosphatase enzyme, which possesses two FLAG markers per dimer molecule. The possible divalent nature of the FLAG epitope-tagged enzyme could make it more resistant to peptide elution. However, efficient elution was observed using only a several-fold molar excess of FLAG peptide. Moreover, we have used FLAG peptide to elute two other proteins from anti-FLAG M2 affinity columns with FLAG peptide, and we found that varying the flow rate during elution from 0.3 to 2.0 mL/min had no appreciable effect on the elution profile (data not shown). Furthermore, in an additional experiment (data not shown), we found the effective binding capacity of the anti-FLAG M2 affinity gel for C-terminal FLAG-BAP to be 41% of the theoretical binding capacity (assuming divalent C-terminal FLAG-BAP). Therefore, we proposed that peptide elution of FLAG fusion proteins from anti-FLAG M2 affinity gel will be a generally applicable and useful procedure.

A



B

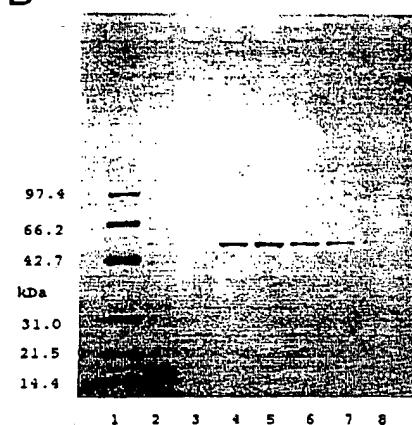


Figure 3. (A) Coomassie blue-stained SDS-polyacrylamide gel of Met-FLAG-BAP column fractions eluted with FLAG peptide. Lane 1, N-terminal FLAG-BAP fusion protein; lane 2, molecular weight standards; lane 3, 2.5-μL column flow through; lanes 4–9, 2.5-μL column fractions 1–6 (see legend of Figure 2), respectively. (B) Coomassie blue-stained SDS-polyacrylamide gel of C-FLAG-BAP column fractions eluted with FLAG peptide. Lane 1, molecular weight standards; lane 2, 2.5-μL column flow through; lanes 3–8, 2.5-μL fractions 1–6 (see legend of Figure 2), respectively.

In addition to purification of N-terminal Met-FLAG and C-terminal FLAG fusion proteins, the anti-FLAG M2 monoclonal antibody can be used for purification of N-terminal FLAG fusion proteins (data not shown). However, the anti-FLAG M1 monoclonal antibody binds to N-terminal FLAG fusion proteins in a calcium-dependent manner, and bound fusion proteins can be eluted using chelating agents (14). Although the binding of the anti-FLAG M2 antibody is not calcium-dependent, it can bind to the FLAG epitope when additional amino acids are found N-terminal to the FLAG sequence. Use of the anti-FLAG M2 affinity gel coupled with peptide elution therefore extends the applications of the FLAG system to purification of FLAG fusion proteins expressed in the cytoplasm (MET-FLAG) and C-terminal FLAG fusion proteins, and it offers an alternative to the extreme conditions often required for elution of antigens from immunoaffinity columns (16).

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Address correspondence to Bill L. Brizzard, Scientific Imaging Systems, Eastman Kodak Company, 25 Science Park, New Haven, CT, USA. Internet: kodak@tigger.jvnc.net

Janet Elizabeth Fulton,  
Eileen Lenore Thacker,  
Larry Dean Bacon and  
Henry Donald Hunt

USDA-Agricultural Research  
Service, Avian Disease and  
Oncology Laboratory, East Lansing,  
USA

## Functional analysis of avian class I (BFIV) glycoproteins by epitope tagging and mutagenesis *in vitro*

Similarities between the physical structures of avian and mammalian major histocompatibility complex (MHC) class I glycoproteins have been proposed based on comparative alignment of their amino acid sequences. To investigate the physical structure of the chicken class I glycoprotein, we cloned the cDNA representing the BFIV locus of the B21 haplotype. A unique, chimeric class I glycoprotein was constructed by incorporating an epitope tag (FLAG) at the N terminus. Monoclonal antibodies to the FLAG epitope served to monitor cell-surface expression for functional analysis of the BFIV21 class I glycoprotein. The chimeric class I glycoprotein was expressed in target cells using an avian leukosis virus (ALV)-derived retrovirus vector (RCASBP). The presence of the FLAG epitope did not interfere with either alloantibody recognition or cytotoxic T lymphocyte interaction. Functional analysis employing site-directed mutagenesis identified BF amino acid residues forming serologic epitopes as well as residues important in antigen presentation to ALV-induced cytotoxic T lymphocytes. BF residues 78 and 81, corresponding to HLA 79 and 82, form an antibody epitope with a slight effect on ALV antigen presentation, consistent with their predicted orientation based on the HLA-A2 crystal structure. Alignment of the BFIV21 sequence with previously published BFIV sequences revealed polymorphisms at position 34 (HLA 34), a monomorphic residue in HLA and H-2. Residue 34 is located in pocket B and is predicted to contact the main-chain carbon of peptides bound in HLA-A2. A site-directed substitution in BFIV residue 34 dramatically alters ALV antigen presentation by the BFIV21 class I glycoprotein. These data indicate that the physical molecular structure of the chicken MHC class I glycoprotein is similar to HLA.

### 1 Introduction

The chicken major histocompatibility complex (MHC) class I molecule is biochemically and functionally similar to the mammalian class I molecule [1]. This 40–42 kDa molecule is expressed on virtually all cells and is thought to play a central role in MHC-restricted antigen presentation to cytotoxic T cells [2, 3]. Six class I loci have been identified, scattered throughout the chicken MHC [4], and there is evidence that more than one locus is expressed [5, 6]. Recent evidence shows that chicken class I loci segregate independently as if they were located on two different chromosomes or separated by a recombinational hot spot [7].

Class I sequences have been determined from several different alleles from both genomic libraries [8] and cDNA [4, 9–11]. The class I sequence presented here is from the B21 haplotype and is of particular interest due to the strong association between this haplotype and resistance to Marek's disease, an avian herpes virus-induced lymphoproliferative disease of chickens [12]. Recently the class I cDNA from the B21 and B13 haplotypes have been expressed and shown to present viral antigens to cytotoxic lymphocytes in an MHC-restricted manner (Thacker, in preparation).

Although the avian and mammalian classes have evolved separately for 270 million years, alignment of the chicken and mammalian class I amino acid sequences suggests that the physical structures are remarkably conserved [4]. Avian polymorphic residues tend to occur in the same regions as in mammalian class I molecules, and residues important for the structural integrity of the  $\alpha$ -helices and  $\beta$ -strands forming the antigen recognition site (ARS) appear to be conserved. While the overall three-dimensional structure of the chicken class I molecule is conserved, there are intriguing differences. For optimal amino acid alignment, gaps must be inserted in the BFIV sequence at three of the  $\beta$ -strand connecting loops, and there is one additional amino acid at the beginning of the  $\alpha$ -helix located in the  $\alpha$ -1 domain. The avian class I glycoprotein has an additional glycosylation site located on the loop connecting the  $\alpha$ -1  $\beta$ -strands 3 and 4. This glycosylation signal disrupts two of the six conserved mammalian intra- $\alpha$ -1 domain salt bridges. There are also regions within the chicken ARS that are polymorphic compared

[I 13953]

Present address: E. L. Thacker, Iowa State University, Veterinary and Research Institute, 1802 Elwood Drive, Ames, IA 50011-1250, USA

Correspondence: Henry D. Hunt, USDA-Agricultural Research Service, Avian Disease and Oncology Laboratory, 3606 East Mount Hope Road, East Lansing, MI 48823, USA (Fax: +1-517-337-6776; e-mail: huntd@pilot.msu.edu)

Abbreviations: ALV: Avian leukosis virus nt: Nucleotide CEF: Chicken embryo fibroblasts ARS: Antigen recognition site

Key words: Avian class I major histocompatibility complex / Cytotoxic T lymphocyte / Mutagenesis *in vitro* / FLAG epitope

with their monomorphic counterparts in mammals. To what extent these differences alter the orientation of amino acid side chains lining or positioned along the chicken class I ARS remains to be determined.

The crystal structure of HLA-A2 [13] has been extremely accurate in predicting the orientation of amino acids which influence antibody epitopes, T cell receptor interactions, and peptide binding in the ARS [14]. Six pockets, distributed throughout the ARS, have been identified as potential anchor sites for peptide side chain binding [14]. Extensive mutation and expression analysis of mammalian class I DNA has identified polymorphic regions that contribute to allele-specific monoclonal antibody binding epitopes [15, 16] and also which residues impact on pocket structure and thus peptide presentation [16, 17]. A similar analysis of chicken class I glycoproteins would provide physical evidence for the theoretical structural similarities which have been proposed for the avian and mammalian class I glycoproteins [4, 11] and provide insight into the orientation of those amino acid residues forming the chicken class I ARS.

To investigate the physical structure of the chicken class I glycoprotein, we have cloned and sequenced the cDNA representing the BFIV locus from the B21 haplotype. To ensure detection of the cloned sequences, a unique epitope tag was attached to the cDNA. With this tag, expression of the BFIV glycoprotein could be monitored even when allo-antisera-binding epitopes were altered. Normal and mutant class I glycoproteins were expressed in the chicken B cell line RP9 using an avian retroviral vector and then used to identify functionally important residues in the chicken class I ARS by both antibody binding studies and <sup>51</sup>Cr-release assays to measure cell-mediated cytotoxic activity. Residues BF 78 and 81, corresponding to HLA 79 and 82, form an antibody epitope consistent with their predicted orientation based on the HLA-A2 crystal structure. This polymorphic region has been identified as an important antibody epitope in both HLA [18, 19] and H-2 [20]. Residue BF 34, corresponding to HLA 34, is polymorphic in the chicken, yet monomorphic in HLA-A,B,C and H-2K,D,L [21–23]. Residue HLA 34 is predicted to influence pocket B of the ARS, and substitutions in residue 34 influence antigen presentation in HLA-A2 [16]. In this study, changes in residue 34 are shown to affect antigen presentation by BFIV, confirming the significance of this residue in peptide presentation.

## 2 Materials and methods

### 2.1 Chicken lines

Chickens used were from either the inbred RPRL 15.B congenic lines [24] or the non-inbred line 0 [25, 26] developed at the USDA Avian Disease and Oncology Lab in East Lansing, Michigan. The congenic lines are 99.9% identical to the inbred 15.B line, but differ at the MHC. The MHC haplotypes used in this study were B2, B5, B15, and B21, and represent common haplotypes found in commercial white Leghorn strains. The B21 sequence was cloned from the 15.N-21 congenic line. The original source of the B21 haplotype was line N from Cornell [27]. Line 0 birds (B21/B21 haplotype) were used as the source of

effector cells for cytotoxic T lymphocyte assays. Chick embryo fibroblasts (CEF) were obtained from 10-day-old embryos from line 0 chickens. LSCC-RP9 (RP9) is a B cell lymphoblastoid cell line induced in a 15.B × 72 chicken (B2/B15 haplotype) by Rous associated virus-2 (RAV-2), a subgroup B avian leukosis virus (ALV) [28].

### 2.2 Enzymatic amplification

The polymerase chain reaction (PCR) conditions used were 200  $\mu$ M of each dNTP, 2.0 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 U AmpliTaq (Perkin-Elmer), with 100 pmoles of each primer and 100 ng of template in a 100  $\mu$ l final volume. Reaction mixtures were overlaid with light mineral oil (Sigma, St. Louis, MO) and placed in an MJ (Watertown, MA) thermocycler for an initial 1 min 96°C denaturation step. This was followed by 30 cycles of amplification (denaturation: 1 min, 95°C; annealing: 1 min, 50°C; extension: 3 min, 72°C) and a final extension of 10 min, 72°C. The subsequent products were then ethanol-precipitated before further manipulation.

### 2.3 Cloning and sequencing

The cloning and sequencing strategies used are described in detail elsewhere [9]. Bursal mRNA was obtained from 15.N-21 B-homozygous chickens and cDNA was produced. Primers specific for the untranslated flanking regions of the BFIV locus were developed using the B12 genomic sequence [8]. These primers, BFIV.5 and BFIV.3 (Table 1) incorporated Hind III and Xba I restriction sites (underlined) and were used with PCR amplification to amplify the BFIV allele from B21 cDNA. The 1274-bp amplification product obtained was cloned into the pRC-CMV eukaryotic expression vector (Invitrogen, San Diego, CA) using the Hind III and Xba I sites. Single-stranded sequence analysis was done in both directions for two separate clones using Tsequencing with 7-deaza nucleotides (USB, Cleveland, OH).

### 2.4 Retroviral vector construction

The avian retroviral vector RCASBP(A) [29] was used to express the BFIV cDNA in the RP9 B cell tumor line. The presence of an endogenous poly(A) signal in sequences cloned into RCAS can interfere with retroviral expression of the inserted sequence. To eliminate this problem, the  $\alpha$ -3 domain of BFIV21 was PCR amplified using primers specific for exon 3 (primer 15.5: nt 511, Table 1 and Fig. 2) and a region 95 bp upstream of the poly(A) site but still within the non-coding region 3' of exon 8 (primer 14.3; nt 1135; incorporating an Xba I site at nt 1155). The subsequent product lacked the endogenous poly(A) signal and was substituted into the Bpu1102 I (nt 615) and Xba I (nt 1155) sites of BFIV21. This modified BFIV21 sequence was used for all subsequent manipulations.

The BFIV21 sequence was transferred into the pUC Cla112N adaptor plasmid using the Hind III and Xba I restriction sites within the poly-linker [29]. It was then cloned into the Cla I site of RCASBP(A) using partial digestion (due to the internal Cla I site in BFIV21).

Orientation of the insert with respect to the retroviral long terminal repeat (LTR) was determined by PCR screening using a primer specific for the LTR of ALV (generously supplied by E.J. Smith) and a BFIV21 internal primer. Fig. 1 shows the orientation and relative location of all primers used for amplification and those restriction endonuclease sites utilized for cloning.

The DH5 alpha *Escherichia coli* strain was used for all transformations. Plasmid DNA was purified by either CsCl<sub>2</sub> density centrifugation or Qiagen plasmid purification columns (Qiagen, Chatsworth, CA).

## 2.5 Incorporation of FLAG epitope

The FLAG epitope tag is an 8-amino acid, highly hydrophilic peptide (NH<sub>2</sub>-DYKDDDDK-COOH) developed by Immunex (Seattle, WA). All eight of these amino acids are required for the binding of the FLAG-specific monoclonal antibody M2 (Kodak-IBI, New Haven, CT). The FLAG coding sequence was introduced into BFIV by PCR amplification using primer BFIV.5, which is specific to the 5' end of BFIV, and primer FLAGM1.3, which is specific to the junction of exons 1 and 2 and also contains the 24-nucleotide sequence encoding FLAG (Table 1 and Fig. 1). The template used was pUCBFIV21. The resulting amplified product (consisting of exon 1, FLAG and 5' end of exon 2) was substituted between the Hind III and Sst I sites of pUCBFIV21 and resulted in the pUCBFIV21FLAG construct. This was subsequently moved into the RCASBP (A) vector as described above.

## 2.6 Production of mutants

The BFIV21 mutants were produced using splicing by overlapping extension [30]. Mutagenizing primers 21M34.5 and 21M34.3 (Table 1 and Fig. 1) were specific for the area to be altered (nt 193 and 195) and contained the desired mutant nucleotide sequence (indicated in bold in Table 1). Each primer was used in PCR amplification in conjunction with a flanking primer (*i.e.* BFIV.5 and 21M34.3 or 14.3 and 21M34.5) in separate reactions with BFIV21FLAG as template. The resulting product of each reaction was isolated by gel electrophoresis from the template and combined for a second PCR amplification. Due to the nature of the mutagenizing primers, these products contain overlapping complementary ends which can anneal. The second PCR amplification utilized the same

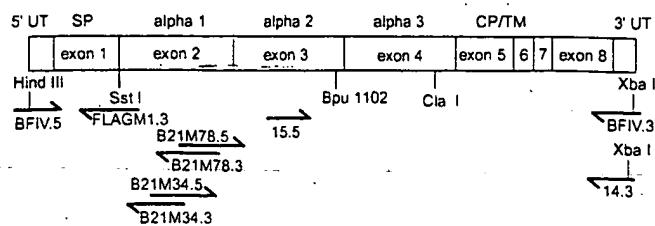


Figure 1. Diagram (not to scale) of BFIV21 cDNA showing location and direction of primers used for amplification and mutagenesis and those restriction endonuclease sites involved in cloning.

flanking primers (BFIV.5 and 14.3) with the annealed product as template. The product from this second PCR amplification was inserted into the Hind III and Bpu1102 I sites of pUCBFIV21 and resulted in the pUCBFIV21M34TFLAG mutant. The amino acid sequence of B21M34T differs from that of B21 only at amino acid 34 in which the M of B21 is altered to T. The pUCBFIV21D78G,R81QFLAG mutant was generated in a similar manner utilizing the 21M78.3 and 21M78.5 set of mutagenizing primers. The B21D78G,R81Q mutant has amino acids G78 and Q81 instead of D78 and R81 found in B21. Each of the mutant constructs were shuttled into the pcDNA3 expression vector (Invitrogen) to obtain single-stranded DNA for sequencing, and into the RCASBP(A) retroviral vector for subsequent expression analysis.

## 2.7 Cell transfactions and infections

All cells were grown in LM media (64% Leibovitz L15, 36% McCoy SA) supplemented with 10% fetal calf serum, 2% tryptose phosphate broth and 1 × 10<sup>5</sup> U penicillin and streptomycin per liter. Transfections into secondary CEF were done using calcium phosphate/DNA coprecipitation. CEF were screened for BF transgene expression within 7 days of initial transfection by either alloantisera or FLAG-specific monoclonal antibody staining and subsequent flow cytometric analysis. RP9 cells were subsequently infected with the various constructs by overnight incubation with supernatant from CEF carrying the RCASBFIV21 constructs. RP9 cells were stained with the FLAG-specific monoclonal antibody M2 (Kodak-IBI) 10 days after infection and positive stained cells were sorted by flow cytometry (FACSort, Becton Dickinson, San Jose, CA). This resulted in the various cell lines used in the antibody binding and cytotoxic T lymphocyte assays described.

Table 1. Sequence of oligonucleotide primers used for cloning BFIV21 cDNA and for site-directed mutagenesis. Restriction endonuclease sites involved for cloning are underlined. Nucleotide alterations including the inserted FLAG sequence are indicated by bold text

Primer	Nucleotide Sequence
BFIV.5	5'GC <del>GGGTACCA</del> <b>AAGCTT</b> CTTGAGAGTCAGCGGTGCGA
BFIV.3	5'GC <del>G</del> <b>TAGAGCGGCCGCT</b> CCCCATCATT <del>T</del> ATTTCAC
15.5	5'CACCAAGAGGAAATGGGAGG
14.3	5'TGCTGGT <b>CTAGACT</b> GTTGGCTCCTTGCAGGC
FLAGM1.3	5'CAGGGTATGGAG <b>GCTCCTTGT</b> CGTCGT <del>C</del> CTTGAGTCGGCCGCCGACAC
B21M34.5	5'CTCTT <b>ACGC</b> ACTACAA
B21M34.3	5'CTGTTGAGTGC <del>GTAA</del> AGAG
B21M78.5	5'AAC <del>TGGGA</del> AA <del>T</del> ACTGC <del>AA</del> CGGGC <del>G</del> GTAC
B21M78.3	5'GCGCCGTTGCAGTATT <del>CCCAGGTT</del>

## 2.8 Fluorescent labeling and detection

Peripheral blood lymphocytes (PBL) for screening anti-sera were obtained from the 15.B congenic lines and were purified with a Histopaque 1077 (Sigma) gradient. Anti-B21 antiserum 7283 was produced by a B2/B15 bird and 7286 was produced by a B5/B15 bird following multiple immunizations with B21/B21 bursal cells.

Cells were incubated on ice with either B21-specific alloantisera (7283 or 7286) or FLAG-specific monoclonal antibody M2 for 30 min. Cells were washed twice and then incubated for another 30 min with either FITC-labeled anti-chicken IgG (Bethyl Laboratories, Montgomery TX) or FITC-labeled anti-mouse IgG, M or A (Cappel, Westchester, PA). Cells were washed three times before flow cytometric analysis.

## 2.9 Cytotoxic T lymphocyte assay

Cytotoxic T lymphocyte (CTL) assays were performed as described by Thacker (in preparation). Briefly, line 0 birds were immunized i.v. with  $2 \times 10^4$  infectious units of RAV-1-SRA (a recombinant subgroup A avian leukosis virus, originally supplied by Dr. H. Robinson) [31]. PBL from 2-5 birds were isolated on a Histopaque-1077 gradient 10 days after immunization, pooled and washed, and then used as effector cells. Target cells were RP9 cells expressing either B21, B13, B21D78G, R81Q or B21M34T class I sequences as described above in addition to the endogenous B2 and B15 haplotypes. These target cells also express ALV viral products due to the RCAS transfection. Effector cells were incubated with  $^{51}\text{Cr}$ -labeled target cells for 4 h at effector-to-target (E:T) ratio of 100:1, 50:1, 25:1 and 12:1. Cytotoxicity, as measured by  $^{51}\text{Cr}$  release from the target cells, was calculated as a proportion of the maximum released following NP-40 lysis of labeled target cells. Percent lysis =  $[(E-S)/(M-S)] \times 100$ , where E=cpm experiment, S=cpm spontaneous, and M=cpm maximum.  $^{51}\text{Chromium}$  release was determined from duplicate samples with a gamma counter (Micromedic). Spontaneous release was determined from cells incubated with medium only, and maximum release was obtained from cells lysed with 1% detergent.

### 3 Results

### 3.1 Nucleotide sequence of BFIV21

The nucleotide sequence of the cloned 1274 bp BFIV21 cDNA is given in Fig. 2. The sequence contains a portion of the 5' untranslated (UT) region, exon 1 which encodes the signal peptide, exons 2, 3 and 4 which encode the  $\alpha$ -1, 2 and 3 domains, exons 5, 6, 7 and 8 which encode the transmembrane and cytoplasmic domains, and a portion of the 3' UT region including the polyadenylation tail. Both of the BFIV21 cDNA clones sequenced have the alternate exon 7.

The predicted amino acid sequence of BFIV21 was aligned with the BFIV12 [4] and BFIV19v1 [11] sequences (Fig. 3). The polymorphic amino acids occur primarily in the  $\alpha$ -1 and  $\alpha$ -2 domains (20.5 % and 15.4 % polymorphic, respectively), with the  $\alpha$ -3, transmembrane and cytoplasmic domains being relatively monomorphic (<2 % polymorphism overall). The human HLA-A2 amino acid sequence is shown aligned with BFIV, with gaps inserted in either BF or HLA-A2 for maximal alignment. The glycosylation site (CHO) found in both chicken and mammalian class I glycoproteins is identified. An additional glycosylation site at BF N37 was found for all three BFIV alleles. This glycosylation site lies within a very conserved stretch of amino acids, and is probably a conserved feature of the BF glycoprotein.

### 3.2 Expression of FLAG epitope and BFIV21

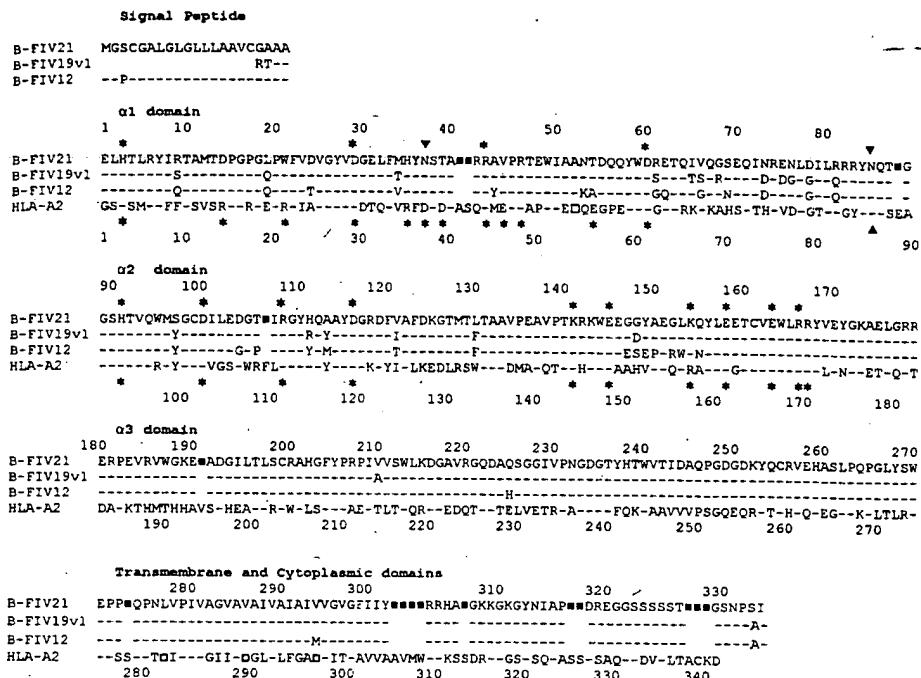
The nucleotide sequence for the FLAG epitope was engineered onto the 5' end of the BFIV21 cDNA sequence at the junction between exon 1 (signal peptide) and exon 2 (alpha 1 domain) of BFIV. Expression of the resulting FLAG-BFIV fusion protein on cells was examined by both FLAG-specific monoclonal antibody M2 and BF-specific alloantisera (Table 2). The FLAG epitope was detected on the cell line expressing the chimeric FLAG: class I (RP9-BFIV21FLAG) glycoprotein, demonstrating expression of the fusion protein. Cell lines expressing the construct lacking the FLAG epitope were not stained, confirming the specificity of the M2 monoclonal antibody. Expression of

```

 HindIII + + + + + exon 1 + + + + +
 1 AAGCTTGTGAGAGTGCAGCGGTGGGAGGCCATGGGCTCGTGGGGGGCGCTGGGCTTGGGGCTGCTGCTCGCCGCGTGTGGGGGGCGC
  * exon 2
 91 GGCGCAGCTCCATACCCCTCGGGTACATCCGTACCGCGATGACGGATCCGGCCCGGGCTGGCTGGTCTGGACGTCGGGCTACGGGTA
 181 CGGGGAACTCTTCATGCACTACAAACAGCACCGCTCGGGGGCTGTCGGCCGACCCGAGTGGATAGCGGAAACACGGGCCAGCTACTG
 271 GGACAGAGAGACGCAGATCGTACAGGGCAGTGGACAGATAACCGCGAGAACCTGGACATACTGCGGGCGCTACACCAAGACCGGGCG
 361 GTCTCACACAGTGCAGTGGATGTCGGCTGTGACATCCTCGAGGATGGCACCATCCGGGGTATCATCAGGCAGCCCTACGATGGGAGAGA
 451 CTTCGTTGCCCTGCACAAAGGCACGATGACCTTAACTCGGGCAGTTCAGAGGCACTTCCACCAAGAGGAAATGGGAGGAAGGGAGGTTA
 541 TCTCTGGGGGGCTGAAGCAGTACCTGGAGGAAACCTCGCTGGAGGTGGCTGCGGAGATATGGAAATACGGGAAGGGCTGAGCTGGGAGAG
  * exon 4
 631 AGAGCGCACCCGAGGGTGCAGTGGGGGGAGGGAGGCCACGGGATCTGACCTTGTCTGCCCGCTCACGGCTTCTACCCGCCGGCCAT
 721 CGTTGTCAGCTGGCTGAAGGACGGCGCGTGGGGGCCAGGACGCCAGTCGGGGGGCATGTCGACCGCGACGCCAGCTACACACACAC
 811 CTGGGTCAACCATGATGGCCACCCCCGGGGGATGGGGGATGGGGGATGGGGGATGGGGGATGGGGGATGGGGGATGGGGGATGGGGGAT
  * exon 5
 901 GTGGGAGCCGCCACAGCCAAACCTGGTGGCCCATCGTGGGGGGGGCTGTCGGCCATGGGCTGTCGGCCATCGGCTACGTGGTTGGGATT
  * exon 6
 991 CATCATCTACAGCGCCATCGAGGGAAAGGGGAAGGGCTACACATCGGCCCGACAGGGAAAGGGTGGATCAGGACGTCGAGCACAGG
 1081 GAGCAACCCCTCCATCTGAGTGTGCTTCAGCTGCAAGGGACCAACAGTCCACACCCAGCATGGGGTGGTGTGAAATGGGACACAGCCCC
 1071 ATCCCTTGTGACCTCTCAGAGTGTCCCTGTGCTTCAGCTGACTGTTATTCTTGCCGTGACTGCTCTGTGAAATAAAATGATGGGCC
  XbaI
 1261 AGCGCCGGCTCTAGA

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Figure 2. Nucleotide sequence of BFIV21 cDNA. Boundaries of the exons are indicated by \*.



the fusion protein was also detectable by the B21-specific alloantisera 7283 and 7286 (produced by B2/B15 and B5/B15 chickens, respectively). Each of these antisera recognize the B21 haplotype expressed on PBL, but 7283 also cross-reacts with PBL of the B5 haplotype (Table 3). Neither antisera cross-reacts with PBL of the B2 or B15 haplotype (present on RP9 cells) or against any other haplotype tested (data not shown). The presence of the FLAG epitope on the amino terminal end of BFIV21 did not interfere with recognition of BFIV21 epitopes by either of these alloantisera.

**Table 2:** Log fluorescent mean channel numbers showing expression on three cell lines of the FLAG epitope as detected by FLAG-specific monoclonal antibody M2 and BFIV21 as detected by B21-specific alloantisera 7283 and 7286

Cell line <sup>a)</sup>	Antibody	
	anti-FLAG mAb M2	B21-specific alloantisera
		7283 7286
RP9-BFIV21	21 <sup>b)</sup>	328 111
RP9-BFIV21FLAG	191	370 160
RP9	20	63 60

a) Cell types are RP9 expressing either BFIV21 or BFIV21FLAG, or non-transfected.

b) Mean log fluorescent channel number.

Table 3 summarizes evidence for the expression of FLAG and B21 epitopes on the cell lines transfected by either RCASBFIV21FLAG or one of the RCASBFIV21FLAG mutants. For simplicity, the cell lines from here on will be referred to as B21, B21M34T or B21D78G, R81Q even though they all also express the FLAG epitope. The FLAG epitope is expressed at a similar level on all three cell lines but is absent on the RP9 cell line, as indicated by binding

**Figure 3.** Predicted amino acid sequence of BFIV21. Shown aligned are the amino acid sequences of BFIV12 [4] and BFIV19v1 [11]. Standard one-letter amino acid codes are used with dashes indicating identity. The BFIV12 sequence is corrected at the signal peptide- $\alpha$ -1 domain junction as described by [9]. The region of the cytoplasmic domain encoded by exon 7 for BFIV12 was obtained from the BFIV12 genomic sequence [8]. Also aligned is the HLA-A2 amino acid sequence (from [20]) with its corresponding enumeration. Insertion of gaps for optimal alignment are shown as █ in BF and □ in HLA sequences. Carbohydrate attachment sites are indicated a triangle (▼) and salt bridges are shown by an asterisk (\*).

of the FLAG-specific monoclonal antibody M2. The B21-specific alloantiserum 7283 recognized all three B21-expressing cell lines. However, it reacted with the B21 and B21M34T cell line at a higher level than the B21D78G, R81Q cell line. This difference in detection was consistent in five assays and was probably due to the lack of a B21-specific epitope recognized by 7283. Adsorption of 7283 antiserum with a B21M34T-expressing cell line removed all B21 specificity. In contrast, adsorption with the B21D78G, R81Q-expressing cell line removed all specificity to B21D78G, R81Q but left considerable reactivity for both the B21 and B21M34T cell lines. The crystal structure model predicts that the side chains of those amino acids altered in B21D78G, R81Q (BF 78 and 81; HLA 79 and 83, see Fig. 4) should point away from the ARS and be available for antibody interaction. Since B21D78G, R81Q lacks only this epitope, adsorption with B21D78G, R81Q should remove all B21 specificity except antibodies specific for this region. Adsorption with RP9 did not alter the B21 specificity of 7283 antiserum. The 7286 antiserum also recognized all three B21-expressing cell lines, but at a lower level than 7283. Adsorption of this antiserum with any of the B21-expressing cell lines (either normal B21 or mutant) removed all B21 reactivity.

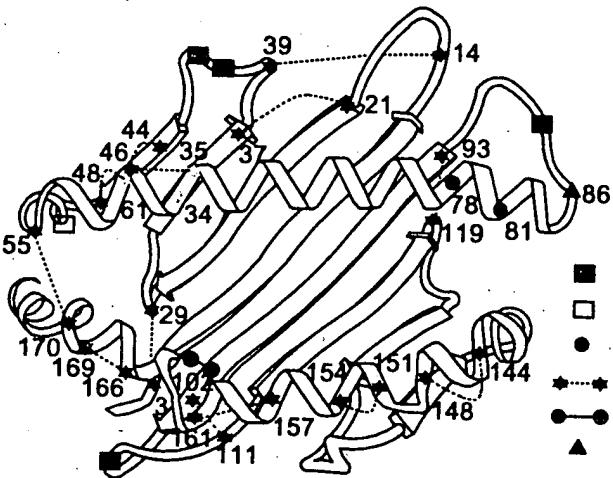
The lower portion of Table 3 shows that alloantiserum 7283 bind to PBL from the B5 haplotype in addition to B21. Adsorption with the B21M34T cell line removed all B21 specificity for PBL (as was seen with the mutant cell lines) and also removed the B5 cross-reacting antibodies. Adsorption with the B21D78G, R81Q cell line removed some of the B21 activity; however, some activity remained for B21 and B5. The alloantiserum 7286, produced by a B5/B15 individual, was not expected to have any B5 specificity. Thus, it reacted with PBL having the B21, but not B5 haplotype. Adsorption of this antiserum with either of the B21 mutant cell lines removed all specificity for B21 PBL, as expected.

**Table 3.** Log fluorescent mean channel numbers showing reactivity of unadsorbed and adsorbed alloantisera 7283 and 7286 on normal and mutant allele-expressing BFIV21 cell lines. Antisera were adsorbed with cell lines expressing either the BFIV21M34T or BFIV21D78G, R81Q mutant construct or the RP9 progenitor cell line

Cell type <sup>a)</sup>	anti-FLAG mAb M2	anti-B21-7283 alloantiserum (2/15) adsorbed with:			anti-B21-7286 alloantiserum (5/15) adsorbed with:			
		None	21M34T	21D78G	RP9	None	21M34T	21D78G
RP9-BFIV21FLAG	565 <sup>b)</sup>	557	35	191	524	233	25	28
RP9-BFIV21M34TFLAG	546	633	47	237	599	284	46	50
RP9-BFIV21D78G, R81QFLAG	525	412	45	37	325	260	44	41
RP9	16	45	37	30	33	36	29	29
PBL-B21	20	526	85	263	424	279	55	66
PBL-B5	20	533	98	299	613	67	68	64
PBL-B15	19	52	58	63	57	58	65	60

a) Cell types are either RP9 expressing the various BFIV21FLAG constructs or non-transfected, or peripheral blood lymphocytes (PBL) of the specified haplotype.

b) Mean log fluorescence channel number.



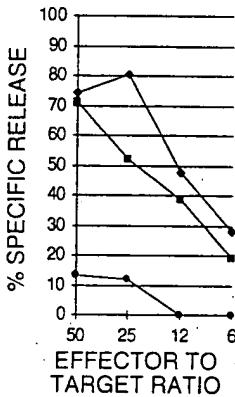
**Figure 4.** Ribbon diagram of HLA-A2 tertiary structure showing conserved mammalian salt bridges and glycosylation site, and location of gaps introduced in either BF or HLA for maximal alignment. Also shown is the predicted location of the two mutants BFIV21M34T (HLA34) and BFIV21D78G, R81Q (HLA79-82). Gray shading indicates that the residue is hidden. ■: Gap in BF; □: gap in HLA; ●: mutation; ★---★: salt bridge; ○---○: disulfide bond; ▲: N-linked glycan.

### 3.3 $^{51}\text{Cr}$ -release assay

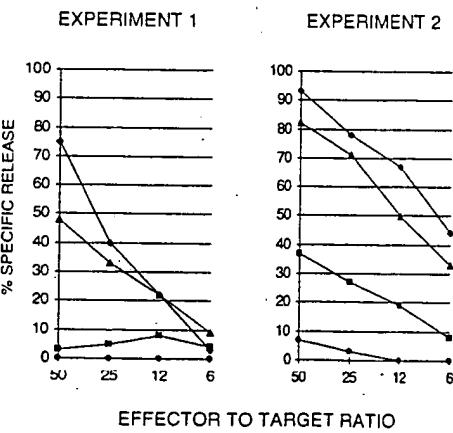
A CTL assay was done using RP9 target cells expressing either BFIV21 or BFIV21FLAG to determine whether the FLAG epitope interfered with the ability of BFIV to interact with the T cell receptor. RP9 cells expressing a non-MHC-matched class I (BFIV13FLAG) were used as a negative control (Fig. 5). The CLT assay was repeated several times with the same results, *i.e.* FLAG did not affect lysis by CTL.

Fig. 6 shows the results of two independent CTL assays using the RP9 target cell lines expressing one of the BFIV21, BFIV13, B21M34T or B21D78G, R81Q class I glycoproteins. Effector cells were PBL obtained from ALV-infected birds of B21/B21 haplotype. At each E:T ratio, there was lysis of the BFIV21- and B21D78G, R81Q-

expressing target cells as indicated by the percent specific release of  $^{51}\text{Cr}$ ; however, lysis of B21D78G, R81Q target cells was consistently lower. The BFIV13 target cells represent an MHC-incompatible negative control and showed very low or no lysis. The target cells expressing the B21M34T mutant showed almost no lysis in one assay and a low level of lysis in the second assay. While these results show variation between assays, the trends are consistent:



**Figure 5.**  $^{51}\text{Cr}$  release assay showing cytotoxicity of ALV-induced T cells against RP9 target cells expressing ALV and either BFIV21 (■), BFIV21-FLAG (◆) or BFIV13-FLAG (○) class I sequences.



**Figure 6.**  $^{51}\text{Cr}$  release assay showing cytotoxicity of ALV-induced T cells against RP9 target cells expressing ALV and either BFIV21 (◆), BFIV13 (○), BFIV21M34T (■) or BFIV21D78G, R81Q (▲) class I sequences.



cally identical to B21. The results of the CTL assay clearly show the significance of residue 34 in peptide binding. The change of M to T in the B21M34T mutant greatly decreased the ability of this cell line to be a target for CTL.

The alignment of the BFIV nucleotide sequences with HLA-A2 and the structure/function data presented show some interesting similarities between the two molecules. Those amino acids predicted from crystallographic studies to be important for the overall structure of the glycoprotein (such as  $\alpha$ -helix or  $\beta$ -strand formation) are conserved [4, 11, 14] and polymorphisms occur in similar amino acids, suggesting similarity in functional significance. However, there are also some intriguing differences between the avian and mammalian class I molecules. In the BF  $\alpha$ -1 domain, only two of the six conserved mammalian salt bridges (HLA H3-D29 and R42-D60) occur. The change in sequence to an additional glycosylation site at BF N37 (HLA D37) interferes with two of the conserved mammalian salt bridges. The degree of amino acid homology between the avian and human  $\alpha$ -1 domains is 40%. These differences suggest structural variation. The presence of conserved salt bridges in mammalian class I molecules has been proposed to be important for stability of the three-dimensional structure [14]. The lack of these salt bridges in the avian class I molecule with conserved structural similarity implies that these salt bridges are not necessary elements for functional class I structures. In contrast to the  $\alpha$ -1 domains, avian and mammalian  $\alpha$ -2 domains are much more similar. There is a 55% amino acid homology between avian and human sequences. The intradomain disulfide bond is conserved, as are all five of the conserved mammalian salt bridges. The  $\alpha$ -helices of the  $\alpha$ -2 domains are important contact sites for the T cell receptor [33], and perhaps this requirement and the rigidity imposed by the intrachain disulfide bond requires more conservation and inflexibility within the  $\alpha$ -2 domain. While fine structural differences may exist, this mutant analysis shows that structurally and functionally, avian and mammalian class I glycoproteins are very similar.

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